

**Identification of lipid mediators using
Drosophila to dissect function and role in
inflammatory disease**

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A thesis presented to the University of Birmingham in for the degree
DOCTOR OF PHILOSOPHY

College of Medical and Dental Sciences
The University of Birmingham
September 2013

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Abstract

Inflammation is widely accepted as an initiator, and involved in the exacerbation of many diseases. Key modulators of inflammatory responses are oxygenated metabolites of polyunsaturated fatty acids (PUFAs). There are two main classes of PUFAs, Omega-3 (n-3) and Omega-6 (n-6). Arachidonic acid, a derivative of omega-6 is known to produce pro-inflammatory mediators known as prostaglandins (C20, n-6) via cyclooxygenase (COX). COX (a member of the myeloperoxidase (MPO) family) is a major therapeutic target of intervention in inflammatory disease. We investigated whether *Drosophila* could be used to model and dissect new novel targets of inflammation. Using an inflammatory model based on a JAK temperature sensitive, gain-of-function mutation (*hop^{Tum}*), we have characterised lipid mediator-signaling pathways in *Drosophila*. Dietary supplementation with PUFAs was able to modulate inflammation, with omega-6 fatty acids increasing, and omega-3 fatty acids reducing, the severity of the *hop^{Tum}* inflammatory phenotype. While COX and MPO inhibitors were able to reduce inflammation, LC-MS analysis of *Drosophila* extracts failed to detect the existence of prostaglandins or their precursor, arachidonic acid (C20). Interestingly, however, we have been able to detect C18 oxygenated metabolites known as hydroxy-octadecadienoic acids (HODEs). HODEs are generated by the oxidation of omega-6 (C18, n-6) fatty acid, linoleic acid. Chiral analysis via LC-MS indicated a higher proportion of the enzymatic chiral form is generated of both 9- and 13-HODE in *Drosophila* samples. Members of the MPO family are able to produce HODEs. We have identified 3 candidate *Drosophila* COX/MPO homologues:

Pxn, Pxt and Irc that may generate HODE inflammatory lipid mediators. We have shown them to have both COX and MPO activity *in vitro*. Deletions of these genes reduced the inflammatory phenotype in the *hop*^{Tum} assay. Functional characterization using deletions of these genes showed affects on longevity as well as survival upon infection. We show that *Drosophila* could be used to investigate 9- and 13-HODE inflammatory effects in diseases such as atherosclerosis.

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Scientific Abbreviations

| | |
|---------------|---|
| % | percent |
| AA | arachidonic acid |
| ALA | α -linolenic acid |
| <i>aPKC</i> | atypical protein kinase C |
| <i>cd</i> | <i>cardinal</i> |
| cDNA | complementary deoxyribonucleic acid |
| <i>cher</i> | <i>cheerio</i> |
| <i>chn</i> | <i>charlatan</i> |
| CIP | calf intestinal phosphatase |
| COX | cyclooxygenase |
| <i>crq</i> | <i>croquemort</i> |
| DAPI | 4,6-Diamidino-2-Phenylindole, Dihydrochloride |
| ddNTP | Dideoxyribonucleotide |
| <i>dDuox</i> | <i>Dual oxidase</i> |
| DHA | docosahexaenoic acid |
| dNTP | deoxyribonucleotide triphosphate |
| Dox | doxycycline |
| <i>DoxA1</i> | <i>prophenoloxidase A1</i> |
| <i>DoxA3</i> | <i>prophenoloxidase A3</i> |
| DTT | dithiothreitol |
| <i>Egr</i> | <i>eiger</i> |
| Eip75B | ecdysone-induced protein 75B |
| EPA | eicosapentaenoic acid |
| FBS | fetal bovine serum |
| g | gram |
| <i>g</i> | g-force |
| <i>GAPDH2</i> | Glyceraldehyde 3 phosphate dehydrogenase 2 |
| <i>gcm</i> | <i>glial cells missing</i> |
| Gaq | G protein α q subunit |
| HCL | hydrochloric acid |

| | |
|----------------------------------|--|
| <i>He</i> | <i>Hemese</i> |
| <i>Hml</i> | <i>Hemolectin</i> |
| HODE | hydroxy-octadecadienoic acid |
| <i>hop</i> ^{<i>Tum</i>} | <i>hopscotch tumourous-lethal</i> |
| <i>inaC</i> | <i>no afterpotential C</i> |
| <i>inaE</i> | <i>no afterpotential E</i> |
| <i>lpk1</i> | <i>inositol polyphosphate kinase 1</i> |
| <i>lpk2</i> | <i>inositol polyphosphate kinase 1</i> |
| IPTG | Isopropyl β -D-1-thiogalactopyranoside |
| <i>Irc</i> | <i>Immune regulated catalase</i> |
| <i>ItgαPS4</i> | <i>Integrin αPS4 subunit</i> |
| <i>ItgαPS5</i> | <i>Integrin αPS5 subunit</i> |
| <i>Itgβv</i> | <i>Integrin βv subunit</i> |
| <i>Itp-r83A</i> | <i>Inositol 1,4,5,-tris-phosphate receptor</i> |
| JAK | janus kinase |
| JNK | jun n-terminal kinase |
| KCL | potassium chloride |
| L | litre |
| LA | linoleic acid |
| LOX | lipxygenase |
| <i>Iz</i> | <i>lozenge</i> |
| M | molar |
| m/z | mass-charge-ratio |
| mg | milligram |
| min | minute |
| ml | millimetre |
| ml | microlitres |
| mM | millimolar |
| mm | millilitre |
| mm | micrometre |
| MPO | myeloperoxidase |
| MRM | multiple reaction monitoring |
| mRNA | messenger ribonucleic acid |
| <i>mys</i> | <i>mysospheroid</i> |

| | |
|------------------------------------|---|
| NaOAc | sodium acetate |
| ng | nanogram |
| <i>NimC1</i> | <i>nimrod C1</i> |
| nM | nanomolar |
| <i>norpA</i> | <i>no receptor potential A</i> |
| °C | degree celsius |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PG | Prostaglandin |
| pH | negative log of hydrogen ion concentration |
| <i>Pkc53E</i> | <i>Protein C kinase 53E</i> |
| <i>Pkc98E</i> | <i>protein C kinase 98E</i> |
| <i>PKCδ</i> | <i>protein kinase C δ</i> |
| <i>Plc21C</i> | <i>Phospholipase at 21C</i> |
| pmol | picomole |
| <i>ProPO45</i> | <i>prophenoloxidase 45</i> |
| <i>Pxn</i> | <i>Peroxidasin</i> |
| <i>Pxt</i> | <i>peroxinectin-like</i> |
| <i>rdgA</i> | <i>retinal degeneration A</i> |
| <i>RpL32</i> | <i>ribosomal protein L32</i> |
| rpm | revolutions per minute |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sec | seconds |
| <i>sktl</i> | <i>skittles</i> |
| <i>sl</i> | <i>small wing</i> |
| <i>sPLA2</i> | <i>secretory Phospholipase A2</i> |
| <i>srp</i> | <i>serpent</i> |
| STAT | Signal-transducer and activator of transcription protein |
| TBS | tris-buffered saline |
| v/v | volume per volume |
| w/v | weight per volume |
| <i>α-Tubulin</i> | <i>α-Tubulin at 84B</i> |
| μ g | microgram |
| μ M | micromolar |

1. Introduction

Inflammatory disease is fast becoming a major problem in the 21st century. Ageing is a key component of this. By 2020, a fifth of the population will be over 65 years of age (World health organisation) – and although individuals are living longer, the quality of life diminishes in later years due to a surge in inflammatory diseases (Franceschi et al., 2000). This is accomplished by changes in lifestyle. Diet has been shown to play a role in the development and severity of inflammatory diseases, especially in the initiation of disease (Hibbeln et al., 2006).

The process of inflammation is an important response to infection and mediates tissue repair. It is a normal biological process that enables the movement of leukocytes to areas of injury (Ley et al., 2007). Without this response, healing would not occur. Most inflammatory responses are acute: they are short-lived and naturally resolve. However, these responses can become uncontrolled and persistent, leading to chronic inflammation that causes tissue damage resulting in disease like rheumatoid arthritis (McInnes and Schett, 2007). The mainstay of current therapeutic intervention is non-steroidal anti-inflammatory drugs (NSAIDs) that blocks the production of pro-inflammatory lipid mediators (Vane, 1971). While these work for many patients, a sub-set of patients does not respond driving the requirement to innovate new defined drug targets.

Key players in inflammation are lipid inflammatory mediators that elicit immuno-mediated responses. Immunity is an essential mechanism required for the survival of most organisms. It protects the organism from invading pathogens such as fungi, bacteria, viruses, and parasites. There are two aspects of immunity, innate and

adaptive (Bell, 2002). Innate immunity is the initial immediate response to detect and clear the invasion of a foreign body. Innate immunity is highly conserved and can be found in most, less primitive forms of life at different degrees of complexity (Beutler, 2004). In contrast, adaptive immunity provides a memory so that when the organism encounters the pathogen again, it will be able to handle the infection more quickly and effectively (Litman et al., 2010). This form of immunity is absent from invertebrates.

Inflammatory mediators orchestrate the responses of both humoral and cellular immunity. It provides another level of control to ensure integration of immune responses. When this level of control is not maintained, immune responses become deregulated and inflammatory disease can occur. Atherosclerosis and rheumatoid arthritis are two prime examples of inflammatory disease that require new innovative therapeutic treatments. We wanted to use *Drosophila* as a model for chronic inflammation, whereby it would allow us to dissect downstream lipid mediator signalling pathways that potentiate inflammatory disease.

1.1 *Drosophila* innate immunity

Drosophila melanogaster, better known as the fruit fly, have only an innate immunity. Like humans, *Drosophila* innate immunity can be divided into cellular and humoral functions (Bulet, Hetru, Dimarcq, & Hoffmann, 1999; Lanot, Zachary, Holder, & Meister, 2001). The cellular component involves cell-mediated actions such as phagocytosis, and encapsulation. In contrast, humoral refers to the actions of secreted macromolecules like antimicrobial peptides (AMPs).

1.1.1 Cellular Immunity

The *Drosophila* immune system is composed of three types of blood cells that all differentiate from a haematopoietic progenitor stem cell known as a prohaemocyte.

From this prohaemocyte, three types of haemocytes are generated: plasmatocytes, crystal cells and lamellocytes (Lanot et al., 2001). As shown in Figure 1, each haemocyte lineage can be defined and distinguished according to the transcription factors they express (Evans et al., 2003). Haematopoiesis occurs at two distinct periods in development. These stages are referred to as embryonic and larval.

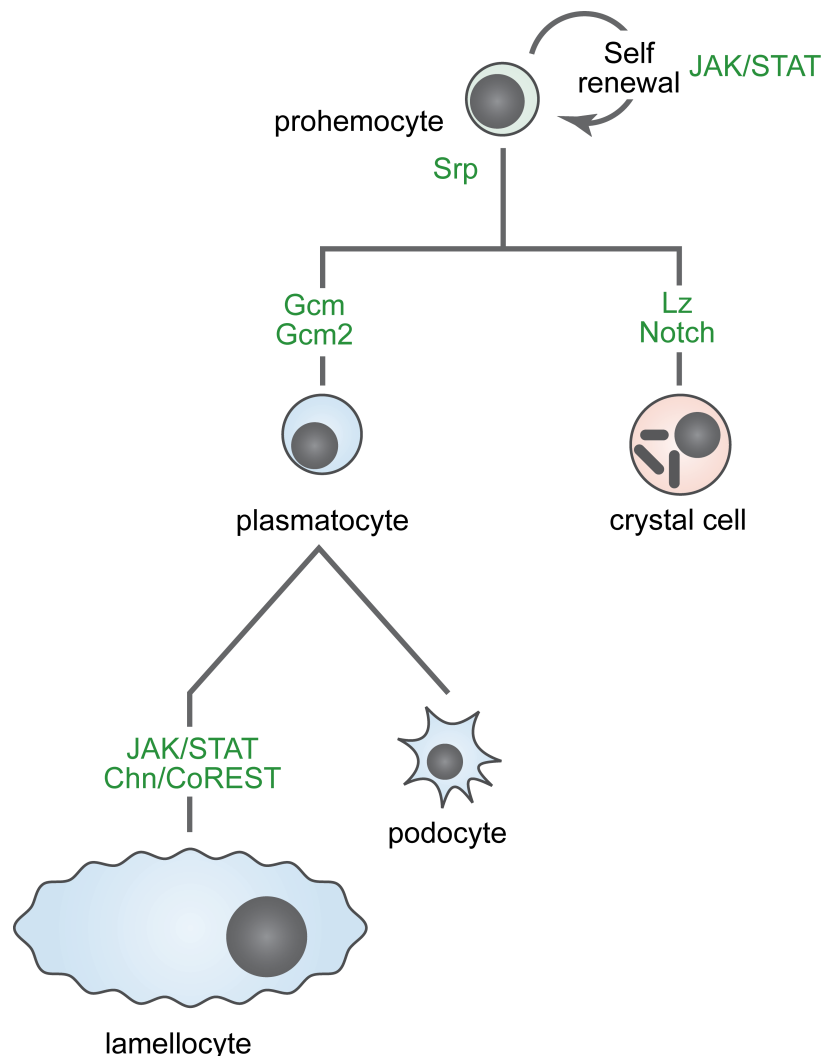


Figure 1. *Drosophila* haematopoietic lineages

Serpent (Srp) specifies the prohemocyte, the haematopoietic progenitor. Expression of Glial-cells missing (Gcm) and Gcm2 favours the plasmatocyte, that can differentiate into lamellocytes via JAK/STAT signalling and Chn, and a number of other cell types such as podocytes and macrophages. Expression of Lozenge (Lz) and Notch signalling results in crystal cell formation. (Figure adapted from Badenhorst, In press.)

The embryonic stage of haematopoiesis occurs in the head mesoderm of the embryo (Mori, 1979). The primary cells produced here differentiate quickly from prohaemocytes and distribute throughout the embryo (Tepass et al., 1994). It is noticeable that this first stage produces mainly plasmatocytes, which are involved in clearing the apoptotic cells that build up during the development of the embryo. Nevertheless a small number of crystal cells are also produced, but at this stage, lamellocytes are not present (Fossett et al., 2003). In the second stage of haematopoiesis, termed the larval phase, the development of the haemocytes occur in the lymph gland which is produced during embryonic development from cells that migrate from the lateral mesoderm (Shrestha and Gateff, 1982). In the lymph gland, all three types of cells are produced, although lamellocytes are rare under normal circumstances, but their production is rapidly increased in the case of parasitic infection (Sorrentino et al., 2002). The two stages of haematopoiesis have been compared to vertebrate haematopoiesis where there are also two stages present called primitive and definitive (Zon, 1995).

Unlike mammals, *Drosophila* lack blood vessels, but instead have an open circulatory system whereby organs are bathed in haemolymph. Haemocytes are circulated in the haemolymph via contraction of the dorsal vessel, which is a single chambered heart (Miller, 1950).

1.1.1.1 *Drosophila* haemocyte types

Three different cell types are present within the fly: plasmatocytes, crystal cells and lamellocytes, each have a distinct immune function. Haemolymph contains approximately 95% of plasmatocytes and around 5% of crystal cells; lamellocytes as already mentioned are not normally present in healthy, uninfected flies (Nappi & Streams, 1969). Embryonic haemocytes are identified by the expression of GATA factor Serpent (Srp) (Lebestky et al., 2000). Srp is required for specification of the

haemocyte primordium within the mesoderm at early embryonic stages, and in later stages, involved in gene expression during haemocyte maturation (Waltzer et al., 2002). Prohaemocytes express Srp initially before any differentiation or maturation markers are expressed (Jung et al., 2005).

1.1.1.1.1 Plasmatocytes

Plasmatocytes are the most abundant haemocytes present in the fly. They express Glial-cells missing (Gcm) and Gcm2 transcription factors, which are required for plasmatocyte differentiation (Bernardoni et al., 1997). They are comparable to macrophages in vertebrates, in that they carry out a number of processes that include: phagocytosis, secretion of extracellular matrix components, coagulation and melanisation (Jones et al., 1999).

The main function of plasmatocytes is phagocytosis, which is important in the development of *Drosophila* as well as protection against invading pathogens. During embryonic development, apoptotic corpses are generated naturally and are cleared by the plasmatocytes (Tepass et al., 1994). Similarly, at pupal stage, plasmatocytes are responsible for engulfing cells fated for death during metamorphosis (Lanot et al., 2001). In larvae and adult stages they are required to identify and remove foreign bodies like invading pathogens (Charroux and Royet, 2009).

The process of phagocytosis involves the same steps as that seen in vertebrates, i.e. attachment/recognition, uptake, and disintegration (Jones et al., 1999). However, the mechanisms by which plasmatocytes recognise and internalise their target are not fully understood, though screening experiments have identified some genes involved in this process. For instance, the scavenger receptor Croquemort (Crq), a homologue of the mammalian CD36 receptor, has been identified to mediate recognition of apoptotic cells for ingestion (Franc et al., 1996). In terms of identifying pathogens

directly for internalisation, two receptors have been identified containing EGF-like repeats, Eater and Nimrod C1 (NimC1). Both receptors are involved in mediating phagocytosis of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Ablation of both receptors has shown significant impairment in clearance of the pathogens (Kocks et al., 2005; Kurucz et al., 2007). Identification of pathogens is a fundamental step towards eventual internalisation of the pathogen into a phagosome (Stuart and Ezekowitz, 2008).

1.1.1.1.2 Crystal Cells

The crystal cells (termed due to the crystalline structures seen within the cell) require expression of Lozenge (Lz), a homologue of the mammalian RUNX/AML1 transcription factor for crystal cell differentiation (Waltzer et al., 2003). As well as Lz, Notch signalling has also shown to induce the crystal cell lineage (Duvic et al., 2002). Crystal cells are non-phagocytic and play an important role in melanisation. The process of melanisation is vital for wound healing and as a defence against pathogens (Galko and Krasnow, 2004). When stimulated, crystal cells rupture releasing prophenoloxidase, which initiates the melanisation cascade. Prophenoloxidase is subsequently cleaved into its activate state phenoloxidase by prophenoloxidase activating enzyme (PPAE) (Ligoxygakis et al., 2002). Ultimately this cascade leads to the production of melanin, which is deposited at sites of injury or during encapsulation of pathogens where it produces an inert capsule (Lemaitre and Hoffmann, 2007).

There are three prophenoloxidases encoded by *Drosophila* genome encodes: *prophenoloxidase A1* (DoxA1), *prophenoloxidase A3* (DoxA3) and *prophenoloxidase 45* (ProPO45). Both DoxA1 and ProPO45 are expressed in crystal cells, whereas DoxA3 is only expressed in lamellocytes and may participate in melanisation during encapsulation (Irving et al., 2005). The rupturing of crystal cells to release these

prophenoloxidases and other components of the melanisation pathway can be regulated by Jun N-terminal kinase (JNK), Eiger (a tumour necrosis factor homologue) and small Rho GTPases like Rac2 that regulate the cell's cytoskeleton (Bidla et al., 2007).

1.1.1.1.3 Lamellocytes

Lamellocytes are large, flat, adherent cells that are exclusively involved in encapsulation. Several regulators have been identified that activate lamellocyte differentiation. JAK/STAT signalling is one of the regulators, and was implicated by a mutation in the sole *Drosophila* Janus Kinase (JAK) that causes constitutive ligand-independent JAK/STAT signalling, resulting in overproliferation of blood cells and significant lamellocyte differentiation. (Luo et al., 1995). Another regulator is Chn, the *Drosophila* homologue of NRSF/REST. Differentiation of plasmatocytes into lamellocytes, both in the lymph glands and in circulation is induced by Chn (Stofanko et al., 2008). Finally, the transcription factor Collier, has also been shown to regulate lamellocyte differentiation as it is required to produce lamellocytes during parasitisation (Crozatier et al., 2004).

Lamellocytes mediate encapsulation of parasites such as wasp eggs with the aid of plasmatocytes. *Leptopilina boulardi* (*L. boulardi*) are endoparasitoid wasps, whereby the female wasps inject their eggs into the haemocoel of *Drosophila* larvae, where the wasps develop and eventually consume the larva. The wasp eggs are first detected by plasmatocytes, which attach themselves to the egg chorion, and through an autoimmune mechanism cause the differentiation of large numbers of lamellocytes in the lymph gland and differentiation of plasmatocytes to lamellocytes in circulation, the signal of which is unknown (Crozatier and Meister, 2007; Stofanko et al., 2010). Lamellocytes form a multi-layered capsule around the foreign body encapsulating it. There is growing evidence that during encapsulation/melanisation,

the parasite is killed by the production of reactive oxygen species (ROS) and intermediates (Nappi et al., 1995) as NO (nitric oxide) radical levels increase during this action (Carton et al., 2009). The capsule is then melanised to form an inert mass. It is unclear whether it is the result of recruitment of crystal cells or lamellocytes, which mediate the melanisation. As mentioned before, lamellocytes express a prophenoloxidase, DoxA3 though its significance is unknown. It is likely that both cell types conduct melanisation during encapsulation.

1.1.1.2 Humoral Immunity

Drosophila also produce a primitive humoral response in conjunction with cellular immunity that provides vital and effective protection during injury and towards invading pathogens (Lemaitre & Hoffmann, 2007). In response to injury and infection, haemocytes release cytokine-like soluble signals that lead to the secretion of different mediators into the haemolymph from the fat body. These include: clotting factors, opsonins, proteases, and most importantly of all antimicrobial peptides (AMPs) (Vierstraete et al., 2003).

AMPs are relatively small, cationic peptides that are constitutively expressed in barrier epithelia, but are also immune induced causing a systemic immune response (Imler & Bulet, 2005). Around 20 inducible AMPs have been identified and categorised into seven classes: Attacin, Cecropin, Defensin, Diptericin, Drosocin, Drosomycin and Metchnikowin (Imler & Bulet, 2005). Each class has specific activity towards a particular pathogen, for example, Diptericin is effective against Gram-negative bacteria, Defensin is effective against Gram-positive bacteria, and Drosomycin is effective against fungi (Lemaitre & Hoffmann, 2007).

When secreted, AMPs accumulate in the haemolymph. It is believed that AMPs target the cell membrane of pathogens resulting in stasis or lysis (Becker et al.,

2010). However, the full mechanism and actions of AMPs are still poorly understood. It is known that two signalling cascades known as The Toll and the Imd pathway mediate humoral AMP production.

1.1.2.1 The Toll Pathway

The Toll pathway is an evolutionary conserved signalling cascade. Toll is a transmembrane receptor that shares sequence homology with the mammalian Interleukin 1 receptor (Hashimoto et al., 1988). Toll is a cytokine receptor that binds an extracellular cytokine known as Spätzle that is activated by pathogen recognition proteins (Hu et al., 2004). This pathway responds to Gram-positive bacteria, fungi and yeast, leading to the production of antimicrobial peptides such as Drosomycin (Lemaitre et al., 1996).

Recognition of Gram-positive bacteria is mediated through detection of lysine containing peptidoglycan (PGN) through peptidoglycan recognition proteins (PGRPs), PGRP-SD and PGRP-SA (which also forms a complex with Gram-negative binding protein 1 (GNBP-1) (Michel et al., 2001)). Yeast detection relies on GNBP-3 and detection of fungi relies on a serine protease known as Persephone (Psh) (Ligoxygakis et al., 2002; Gottar et al., 2006). Once the recognition molecules have bound pathogen components, they activate Spätzle processing enzyme (SPE). SPE proteolytically converts pro-Spätzle into activated Spätzle, which binds to Toll and induces dimerisation of the receptor (Weber et al., 2003). Dimerisation of Toll causes the recruitment of the adaptor protein Myd88 which interacts with the Toll Interleukin 1 receptor domain (Tauszig-Delamasure et al., 2002). This in turn recruits Tube and Pelle (IL-1 receptor-associated kinase (IRAK)), which forms a complex with Myd88 that is mediated through their death domains (Towb et al., 2001). This complex then causes the dissociation of the NF- κ B homologues Dif/Dorsal from

Cactus ($\text{I}\kappa\text{B}$) by phosphorylation via Pelle's (IRAK) kinase activity (Huang, Chen, Kunes, Chang, & Maniatis, 2010). Dif/Dorsal can then freely migrate into the nucleus and elicit transcriptional changes such as induction of AMP genes (Kidd, 1992).

1.1.2.2 The IMD Pathway

In contrast to the Toll Pathway, the Imd (Immune deficiency) pathway responds to Gram-negative bacteria through the production of AMPs such as Diptericin. This pathway however is not as well characterised as the Toll pathway; though it was first identified in flies due to a mutation called *immune deficiency (imd)* (Corbo & Levine, 1996). These mutant flies were more susceptible to Gram-negative pathogens, indicating that this pathway was important in the response towards this type of pathogen (Lemaitre et al., 1995).

Activation of the pathway relies on recognition of peptidoglycan (PGN) found in bacterial walls, especially components of Gram-negative bacteria PGN like meso-diaminopimelic acid (mesoDAP) and tracheal cytotoxin (TCT) (Kaneko and Silverman, 2005). The recognition of PGN is mediated by the transmembrane receptor PGRP-LC and intracellular membrane PGRP-LE (Kaneko et al., 2006; Choe et al., 2005). PGRP-LC encodes three alternatively spliced transcripts: PGRP-LCa, LCx and LCy (Leulier et al., 2003). These three receptor variants have unique extracellular recognition domains (Werner et al., 2000). Binding of Gram-negative peptidoglycan by the PGRPs results in the recruitment of Imd (Corbo & Levine, 1996). This leads to a signalling cascade that results in the cleavage of Relish via the caspase Dredd (Leulier et al., 2000). Cleavage of Relish produces an N-terminal Rel homology domain (RHD) transcription factor, which translocates into the nucleus resulting in up-regulation of AMPs that help protect against Gram-negative bacteria (Ertürk-Hasdemir et al., 2009).

1.2 Lipid derived mediators

In a wider context, both cellular and humoral immunity are orchestrated by lipid-derived mediators, which play a crucial role in many physiological processes. Lipid mediators are utilised as signalling molecules involved in homeostasis, for example, the hormone hydrocortisone produced and regulated by the hypothalamic-pituitary-adrenal axis, to eicosanoids that are involved in inflammation. Lipid molecules that are pro-inflammatory, and more recently, those involved in resolution of inflammation have become important targets of therapeutic intervention for chronic inflammatory diseases like rheumatoid arthritis and atherosclerosis.

1.2.1 Polyunsaturated fatty acid (PUFA) precursors

Polyunsaturated fatty acids (PUFAs) are the known initial precursors to the abundant immuno-modulatory lipid mediators. PUFAs are long chain fatty acids that contain two or more double bonds in their carbon backbone. There are two main PUFAs, omega-3 and omega-6 that are classified by the location of the last double bond from the end terminal methyl group, n-3 (omega-3) and n-6 (omega-6). Many PUFAs, for example linoleic acid (18:2 n-6) and α -linolenic acid (18:3 n-3) cannot be synthesised *in-vivo* in animals, and so must be obtained from the diet making them essential fatty acids (Das, 2006).

These essential fatty acids are metabolised further to produce the key intermediates that are used to synthesise immuno-inflammatory mediators. Linoleic acid (C18) can be extended to arachidonic acid (AA: C20), and α -linolenic acid (C18) can be converted to eicosapentaenoic acid (EPA: C20) (Figure 2). Both conversions rely upon the same enzymes; firstly, Δ 6-desaturase removes two hydrogen atoms forming a double bond, followed by the addition of two further carbon atoms by an

elongase, and a further double bond added by $\Delta 5$ -desaturase (Schmitz & Ecker, 2008).

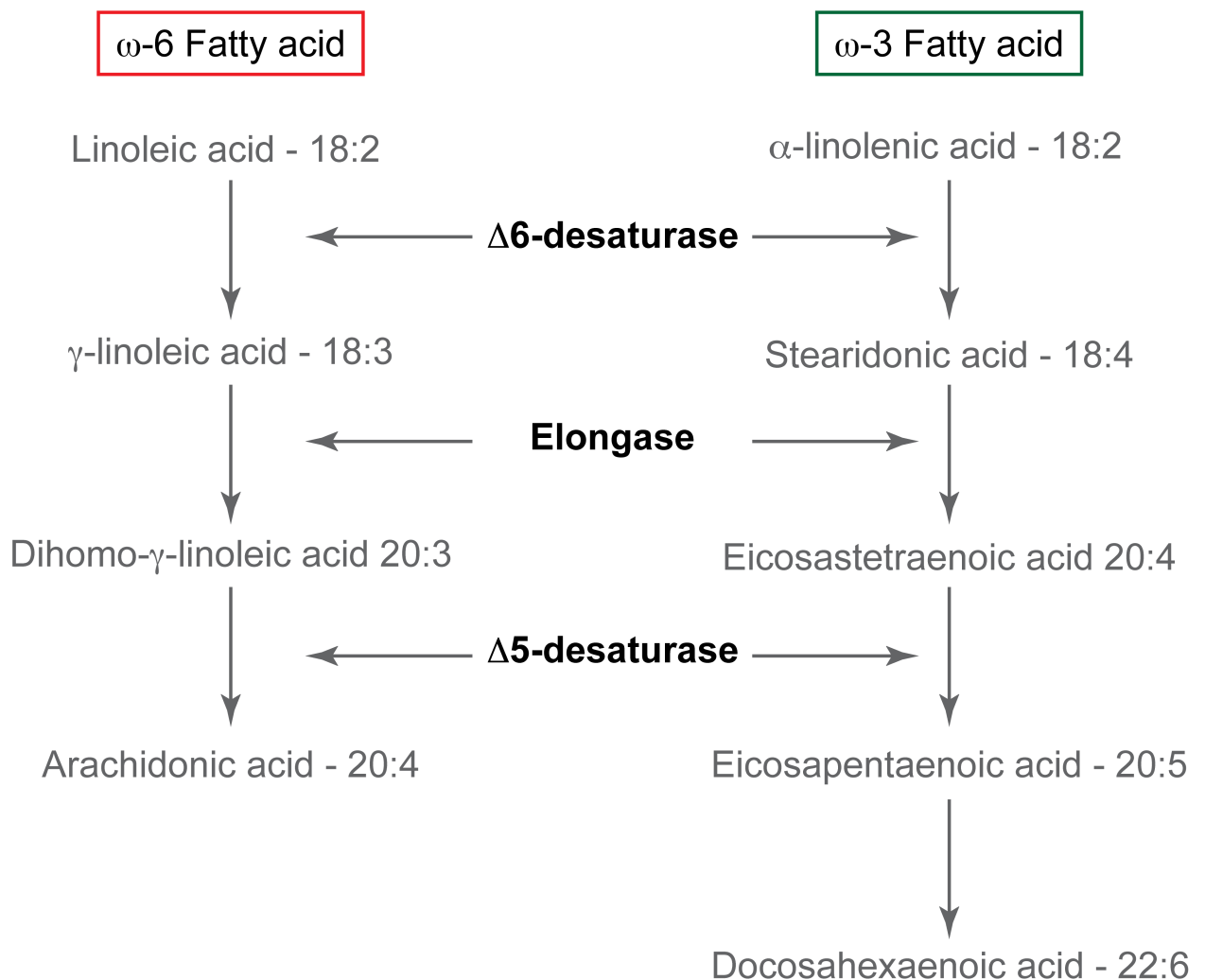


Figure 2. Metabolic pathways of linoleic acid and α -linolenic acid

Omega-6 fatty acid, linoleic acid and omega-3 fatty acid, α -linolenic acid, are essential fatty acids obtained through the diet. They both share the same enzymes, which process them further to the immune-modulatory mediator precursors: Arachidonic acid (AA), eicosapentaenoic acid (EPA) and (Docosahexaenoic acid (DHA).

AA and EPA, as well as their precursors are incorporated and stored in the phospholipid membranes of cells and organelles in an esterified form (Bandeira-Melo et al., 2002). AA is the major PUFA as it is the most abundant C20 PUFA found in phospholipids, but there is very little free AA available. Once incorporated into phospholipid membranes fatty acids can be liberated by several phospholipases. The predominant phospholipase to do this is phospholipase A₂ (PLA₂) which hydrolyses the sn-2 ester bond (Arita et al., 1989) (Figure 3). A pathological and/or physiological stimulus from a cell or tissue drives the activation of PLA₂ (Piper & Vane, 1971).

There are several types of PLA₂s found in mammals that are categorised into four main groups based on their biochemical properties and molecular structure (Six & Dennis, 2000). The first being secretory phospholipase A₂ (sPLA₂) of which there are ten known in mammals. Nine are functional and one is a pseudo gene that is no longer expressed as it has lost its protein coding ability (Tischfield et al., 1996). They function extracellularly and are stimulated by millimolar Ca²⁺ concentrations. The second group are known as cytosolic PLA₂s (cPLA₂s). There are 6 known cPLA₂s. They require Ca²⁺ for catalytic activity and are stimulated by micromolar Ca²⁺ concentrations. Out of the 6 cPLA₂s, cPLA₂α has been shown to be the most important in the production of eicosanoids (Bonventre & Saperstein, 2002). The third group is Ca²⁺ independent PLA₂s (iPLA₂s), which are also found in the cytosol. The final group of PLA₂s inactivate platelet-activating factor (PAF) via PAF acetyl hydrolases (Tjoelker et al., 1995).

Another pathway by which free fatty acid can be liberated from the phospholipid membrane is via phospholipase C (PLC) (Figure 3). The mechanism and action by which PLC liberates free fatty acid was first observed with AA (Iturralde et al., 2005), but many fatty acids can be liberated in this way. When a G-protein coupled receptor

is stimulated, it results in a conformational change to the α -subunit of the trimeric G-protein, causing GDP to dissociate and GTP to bind (Zhu and Birnbaumer, 1996). The α -subunit dissociates from the β and γ subunits, allowing $G\alpha_q$ (the α -subunit) to activate PLC. PLC hydrolyses phospholipid, and in doing so, phosphatidylinositol 4,5-bisphosphate (PIP_2) is cleaved into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG can then be hydrolysed by DAG lipase to produce monoacylglycerol and AA (and other free fatty acids) (Iturralde et al., 2005).

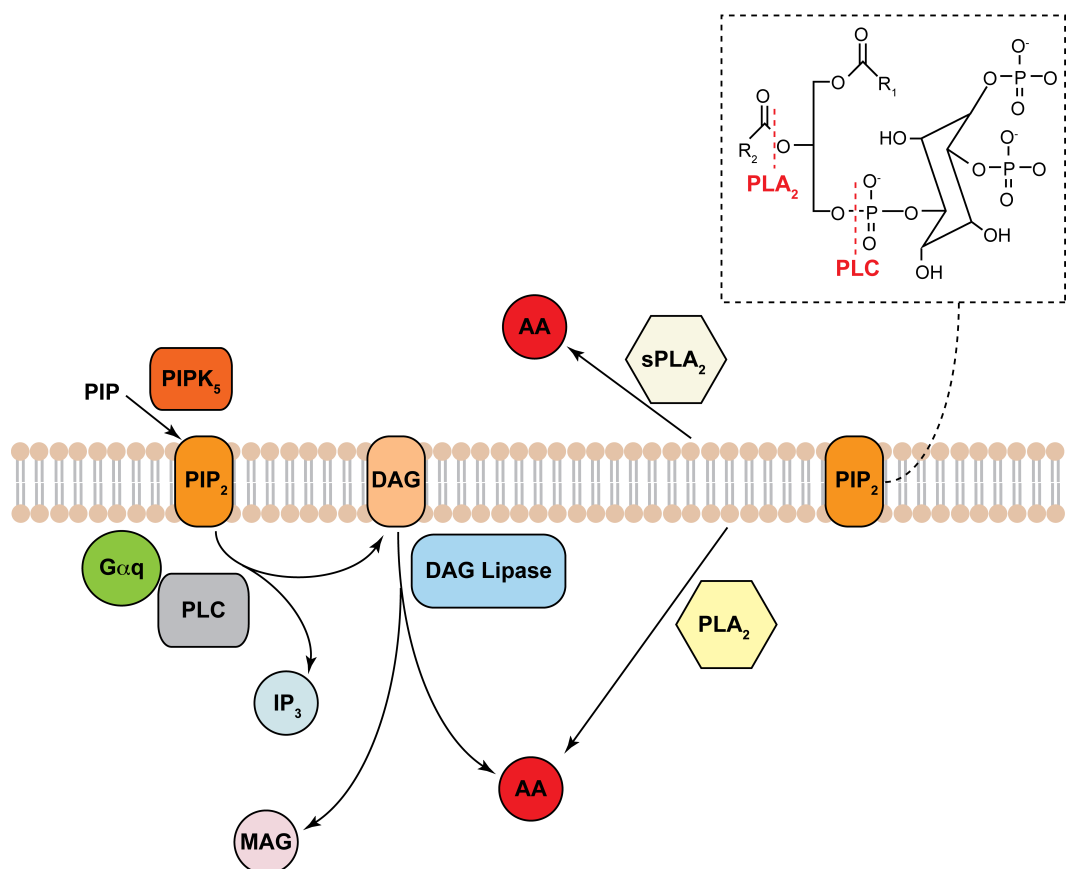


Figure 3. Fatty acid liberating pathways

Free fatty acid, arachidonic acid (AA) in the case of this figure, can be liberated by either phospholipase A₂ (PLA₂) or phospholipase C (PLC). PLA₂ releases free fatty acid by hydrolysing the sn-2 acyl bond of phosphatidylinositol 4,5-bisphosphate (PIP₂). The availability of PIP₂ in the membrane is maintained by phosphatidylinositol phosphate kinase (PIP₅K) that phosphorylates phosphatidylinositol-4-phosphate (PI4P) into PIP₂. Gα_q activates PLC which cleaves PIP₂ just before the phosphate group producing inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG is hydrolysed by DAG lipase, releasing monoacylglycerol (MAG) and free fatty acid (AA).

1.2.2 Pro-inflammatory lipid mediators

The focus of therapeutic targets for inflammation has been predominantly with pro-inflammatory lipid mediators. AA is further metabolised to produce the eicosanoids. Eicosanoids are C20 oxygenated lipid signalling mediators of which there are two main classes. The two classes of eicosanoids are classified by the enzymes that produce them; lipoxygenases (LOX) produce leukotrienes (4 series), which includes both hydroperoxy and hydroxyl-eicosatetraenoic acid (HPETE and HETE), whereas cyclooxygenases (COX) produce the prostanoids consisting of thromboxanes (2-series), prostacyclins and prostaglandins (2-series) (Funk, 2001) (Figure 4). Since the discovery of prostanoids and their involvement in both acute and chronic inflammation, they have been the most widely targeted for therapeutic intervention.

Cyclooxygenases (COX) are members of the larger myeloperoxidase superfamily (Daiyasu & Toh, 2000). They are haem-containing homodimers that have two separate catalytic sites; a peroxidase, which can reduce several hydroperoxides such as hydrogen peroxide, and a cyclooxygenase active site that binds AA (Kulmacz et al., 2003). The two sites work in tandem in a two-step process to produce the prostanoid precursor, PGH_2 . AA inserts into the cyclooxygenase active site, where a Tyr385 residue is oxidised by the haem to become a radical. The Tyr385 radical causes the abstraction of a pro-S hydrogen atom on C13 of AA. This results in the production of the intermediate hydroperoxy endoperoxide (PGG_2) (Rogge et al., 2006). The peroxidase active site then reduces PGG_2 to PGH_2 (van der Donk et al., 2002), the precursor to the prostanoid subclasses.

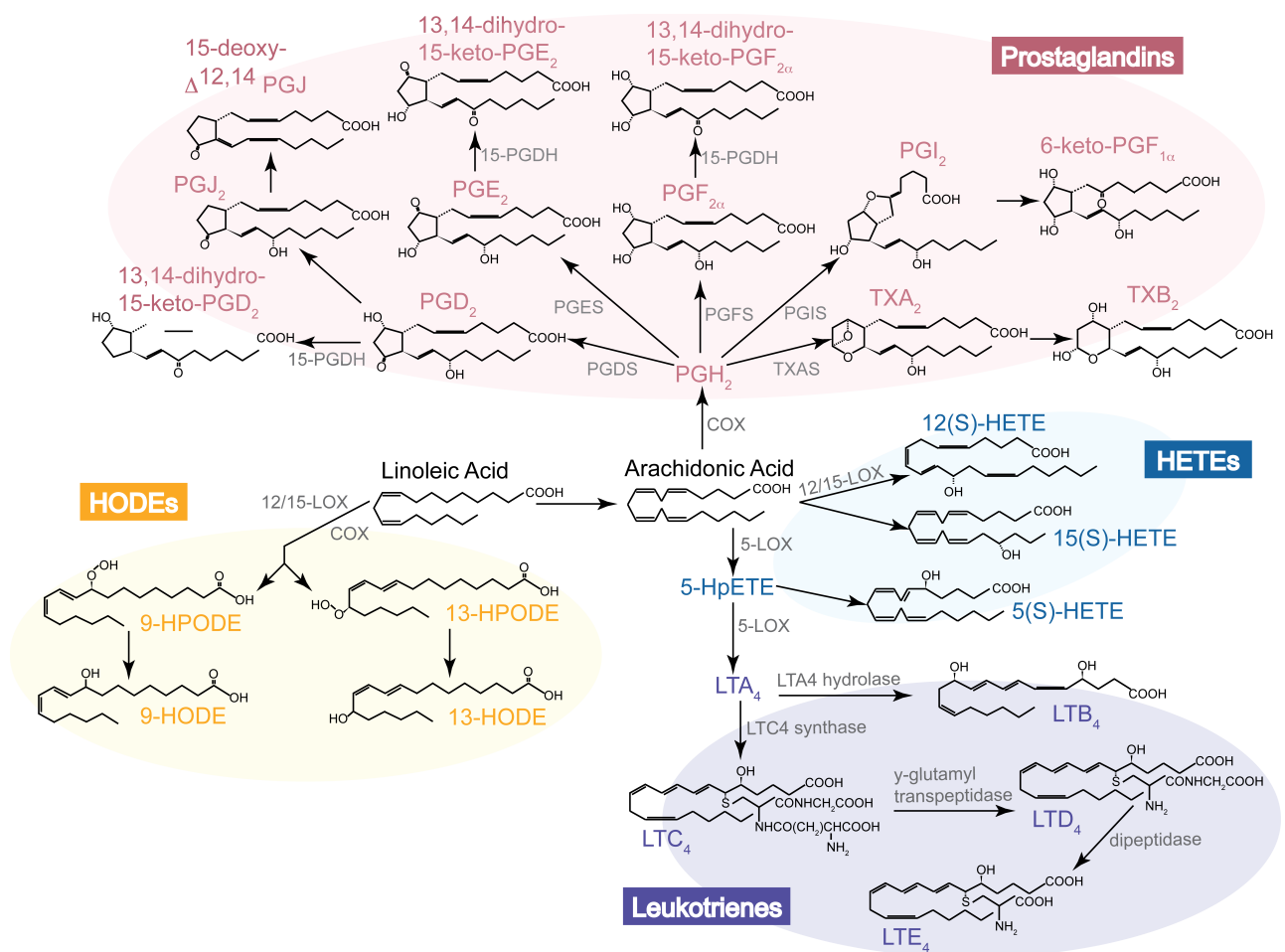


Figure 4. Production of lipid immuno-inflammatory mediators from linoleic acid and arachidonic acid

Linoleic acid can be directly metabolised by either lipoxygenase (LOX) or cyclooxygenase (COX) to produce hydroxy-octadecadienoic acids (HODEs). Arachidonic acid (AA) is processed by COX to produce prostaglandins. Lipoxygenases process AA into the leukotrienes and hydroxy-octadecatetraenoic acids (HETEs). Figure adapted from Shimizu (2009).

There are two COX isoforms, COX-1 and COX-2. It is considered that COX-1 is constitutively expressed in most cells and tissues as it produces prostaglandins that are important for homeostatic functions such as haemostasis. In contrast, COX-2 is an inducible enzyme highly expressed in immune cells and tissues and is up regulated in immuno-inflammatory responses. However, new evidence suggests that both isoforms can be expressed in the same tissues and both may be constitutive and inducible (Zidar et al., 2009).

The main role for both of these enzymes is to convert AA into the precursor PGH_2 . PGH_2 forms the precursor to all the prostanoids: prostaglandins, prostacyclin and thromboxanes. Each subclass enforces a different, yet essential, physiological response. Thromboxane (TXA_2) is a potent vasoconstrictor (Ellis et al., 1976) and aids in the activation of platelets (FitzGerald, 1991). Prostacyclin (PGI_2) on the other hand is a vasodilator and inhibits platelet aggregation, suggestive of a protective role (Rahman et al., 2011). Due to TXA_2 and PGI_2 's conflicting actions, they remain in a homeostatic balance of each other's actions.

The prostaglandins consist of PGD_2 , PGE_2 and PGF_{2a} . PGE_2 is the most studied of the prostaglandins as it is the most abundantly distributed and has a wide range of physiological actions. PGE_2 is involved in all the classic inflammatory cardinal signs like redness, swelling and pain (Legler et al., 2010). PGD_2 on the other hand is predominantly expressed in mast cells where it plays a role in allergen responses (Jowsey et al., 2001). It is also expressed in the central nervous system where it has several neurophysiological actions such as regulating sleep and pain perception (Urade and Hayaishi, 2011). Both PGE_2 and PGD_2 have contrasting actions, as well as having pro-inflammatory effects, they also elicit anti-inflammatory actions that are involved in initiating resolution (Moghaddami et al., 2013; Ogawa et al., 2009). Lastly,

PGF_{2α} like the other prostaglandins has several different actions. PGF_{2α} is well known for its involvement in ovulation and parturition (Darling et al., 1982), but is also involved in acute and chronic inflammation as it has been shown to be elevated in patients with arthritis (Basu et al., 2001).

The synthesis of each specific prostanoid from the precursor PGH₂ is controlled by prostanoid specific synthases (Hinman, 1972). Tissue specific expression of the synthases allows for control and regulation of where the prostanoids are produced. Another level of regulation is through the availability of AA, which is the rate-determining step in the production of eicosanoids. However, expression of these lipid mediators can become dysregulated and lead to a number of chronic inflammatory conditions that include inflammatory bowel disease and breast cancer (Subbaramaiah et al., 2004; Singh et al., 2005). Treatment with non-steroidal anti-inflammatory drugs such as aspirin can alleviate inflammation. These act by inhibiting COX activity and prostaglandin production, making COX an ideal target for therapeutic intervention (Vane, 1971).

1.2.3 Anti-inflammatory lipid mediators

Pro-inflammatory prostaglandins have been at the forefront of the inflammatory field due to their involvement in inflammation (Ricciotti and FitzGerald, 2011). This in part was fuelled by the discovery of aspirin's (a non-steroidal anti-inflammatory drug) actions, which alleviates inflammation through inhibiting COX and preventing production of prostaglandins (Vane, 1971). COX soon became a popular therapeutic target for inflammation. However, prostaglandins were soon discovered to have a wide range of homeostatic functions that are fundamental to an animal's health. Inhibition of COX, although reduces inflammation, it impinges on the housekeeping functions of prostaglandins resulting in adverse affects particularly seen with COX-2

inhibition (Wong et al., 2005). It has therefore become more popular to embrace the naturally produced anti-inflammatory lipid mediators, which can potentially be manipulated to actively resolve chronic inflammation.

During injury or infection, acute inflammation involving the recruitment of leukocytes and release of inflammation-promoting signals such as prostanoids is vital for healing and combating infection. It was initially thought that the resolution of inflammatory responses was passive relying simply on dissipation. Instead, it is an active process that initiates shortly after inflammation-promoting mechanisms have started (Serhan, 2004). With the exception to lipoxin A₄ (LXA₄) and LXB₄, which are produced from linoleic acid, the anti-inflammatory and pro-resolution lipid mediators are derived from omega-3 EPA and a further metabolite of EPA, docosahexaenoic acid (DHA). These particular lipid mediators include resolvin E-series made from EPA, and resolvin D-series and maresins that are formed from DHA.

Lipoxins derived from AA were the first lipid mediators to be identified inflammatory-resolving actions (Serhan et al., 1984). LXA₄ and LXB₄ are synthesised from AA through a series of lipoxygenases predominantly expressed in leukocytes such as neutrophils. They have been shown to antagonise leukotrienes through binding to leukotriene receptors BLT1 and cysLT1 to inhibit the production of pro-inflammatory agents such as IL-8 (Gronert et al., 2001) which halts leukocyte infiltration (Chiang et al., 1999). Lipoxins also have a dedicated receptor, ALX that regulates the production of pro-inflammatory mediators such as TNF α , and prevents oxidative burst (Chiang et al., 2003).

Resolvins were originally identified in mouse resolving-exudates (Serhan et al., 2002). From the exudates, two classes of resolvins were identified, the E-series and D-series resolvins. There are two known E-series resolvins, resolvin E1 (RvE1) and

resolving E2 (RvE2), both of which are synthesised from EPA. EPA is oxygenated via either aspirin treated COX-2, or cytochrome P450 to produce 18-hydroperoxy-EPE which is further oxygenated to 5S-hydroperoxy-18-hydroxy-EPE by 5-LOX which can then be processed to produce either RvE1 or RvE2 (Serhan, 2002).

The second class of resolvins, the D-series, are derived from DHA. DHA can be processed in two ways to produce either R or S stereochemical versions of the D-series resolvins. Utilisation of cytochrome P450 or acetylated COX-2 produces the R enantiomer, whereas 15-LOX produces the S enantiomer. These enzymes produce 17-hydroperoxy-DHA which is processed further to produce the four D-series resolvins, RvD1-RvD4 (Stewart, 2009).

The resolvins have similar actions to the lipoxins. Resolvins prevent transendothelial migration of leukocytes, RvE1 being extremely potent in comparison to dexamethasone (Serhan et al., 2000). This is part can be explained by the down regulation of adhesion molecules such as L-selectin and integrin CD18 seen upon RvE1 treatment (Dona et al., 2008). RvD1 has also been shown to be a potent regulator of leukocyte transendothelial migration (Sun et al., 2007).

Maresins on the other hand are less well characterised. They are produced by macrophages and act directly on macrophages. DHA is utilised by 14-LOX to produce 14S-HpDHA which is further processed to produce maresin 1 (MaR1) (Serhan et al., 2009). MaR1 can reduce leukocyte infiltration by up to 50-80% in zymosan-induced peritonitis, it is a potent enhancer to macrophages in apoptotic leukocyte uptake, and showed tissue regeneration possibilities in the simple flatworm organism planaria, due to accelerated head growth after a head resection (Serhan et al., 2012).

1.2.4 Hydroxy-octadecadienoic acids (HODEs)

Another class of inflammatory oxidised lipids are hydroxyl-octadecadienoic acids (HODEs), which are highly abundant in inflammatory tissues and are now recognised as biological markers of oxidative stress (Yoshida et al., 2008). They are stable oxidative derivatives of LA consisting of 9-HODE and 13-HODE (Figure 4). HODEs can be generated enzymatically, or non-enzymatically from LA. The enzymatic route utilises either lipoxygenase or cyclooxygenase enzymes, which oxygenate LA at either the 9- or 13-carbon, position to produce the intermediate, hydroperoxy-octadecadienoic acid (HPODE). HPODE intermediates are reduced by peroxidase activity to the stable HODE lipid mediator. HODEs can be further metabolised by dehydrogenases to oxooctadecadienoic acids (oxoODE) (Shearer, Harris, Pedersen, & Newman, 2010).

HODEs have a well documented function in atherosclerosis, as they are the most abundant oxidation product found in plaques (Waddington et al., 2003). Studies show that HODE deposition in early lesion formation is predominantly enzymatic, shifting to non-enzymatic at later stages of atherosclerosis (Brinckmann & Kühn, 1997; Waddington et al., 2003). Raised levels of oxidised low-density lipoprotein (oxLDL) occurs in atherosclerosis; 9- and 13-HODE are 20 times more abundant in oxLDL from atherosclerosis patients compared to control patients (Jira et al., 1998).

Recently, structural studies confirmed that 9- and 13-HODE are natural ligands and activators of the peroxisome proliferator activated receptor γ (PPAR γ), showing that they can bind individually or together (Itoh et al., 2008). PPAR γ signalling results in PPAR γ forming a heterodimer with retinoic acid receptor (RXR), which binds to PPAR-response elements that can regulate genes involved in monocyte differentiation and lipid metabolism (Lawrence and Natoli, 2011; Cristancho and

Lazar, 2011). PPAR γ is expressed in both endothelial cells and macrophages. Endothelial cells up regulate ICAM 1 expression through PPAR γ which elicits monocyte adhesion that can be exacerbated further as endothelial cells also synthesise HODEs (Haas et al., 1990; Kaduce et al., 1989).

It has been suggested that 13-HODE has protective properties in atherosclerosis and is actively produced by 15-LOX in macrophages. In early onset atherosclerosis, 13-HODE signalling through PPAR γ results in the up-regulation of the scavenger receptor CD36 which is involved in up take of oxLDL (Jostarndt et al., 2004). Uptake of oxLDL is seen to be protective initially as it removes it from the environment, but too much uptake leads to the action being pro-atherogenic. Though 13-HODE has been shown to also reduce platelet and endothelial cell interactions preventing thrombogenesis (Bertomeu et al., 1990). In the later stages of the disease, the abundance of 9 and 13-HODE overwhelms the potential protective mechanisms and results in increased lipid accumulation and macrophage differentiation into foam cells. The mechanism behind foam cell differentiation is still not fully characterised. Both 9- and 13-HODE have dose dependent potent chemotactic properties towards leukocytes similar to 15-HETE (Henricks et al., 1991). G2A receptor signalling via 9-HODE binding results in the production of pro-inflammatory cytokines such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and causes suppression of cell proliferation in human keratinocytes; the same was not seen with 13-HODE (Hattori et al., 2008). Overall, the physiological effects of HODEs are largely uncharacterised, though the consensus indicates 9-HODE has pro-inflammatory effects, and 13-HODE has anti-inflammatory effects.

1.2.5 Eicosanoids in *Drosophila* immunity

The function of Immuno-modulator lipids in *Drosophila* immunity has not been well studied. However, interest in this field is growing because of the need for new models to explore functions and biosynthetic pathways of these mediators. *Drosophila* provides a genetic model in which to explore these.

Initial data suggested that invertebrates could produce prostaglandins. Studies of the gorgonian octocoral, *Plexaura homomalla* (a Caribbean coral), shown that 2-3% of their dry weight was composed of prostaglandin esters. These were identified as 15R-PGA₂ methyl ester acetate, 15R-PGA₂ methyl ester, and PGE₂ methyl ester (Weinheimer and Spraggins, 1969). A COX-like enzyme was later found that has 50% sequence similarity to mammalian COX and was shown to produce the intermediate prostaglandin PGH₂, which was converted to PGE₂ and then rapidly acetylated to PGA₂ (Valmsen et al., 2001).

The tobacco hornworm, *Manduca sexta*, has also been used widely to study presence and function of eicosanoids. Microsomal-enriched preparations of haemocytes and fat body from *Manduca sexta* larvae were used to detect the presence of endogenous PGA₂, PGE₂, PGD₂, and PGF_{2α} prostaglandins as well as a lipoxygenase product that co-chromatographed with 15-hydroxy eicosatetraenoic acid (HETE) (Gadelhak et al., 1995; Stanley-Samuelson and Ogg, 1994). As yet, the enzymes that synthesise these lipid mediators have not been characterised.

The physiological function of these prostaglandins in *Manduca sexta* has further been analysed. Chemotactic experiments using haemocyte migration towards formyl-methionyl-leucyl-phenylalanine (fMLP) was used to show eicosanoid involvement in haemocyte chemotaxis. Haemocyte migration was reduced when treated with indomethacin (a COX inhibitor), an effect reversed by the addition of arachidonic acid

(Merchant et al., 2008). Eicosanoids have also been suggested to play a role in nodulation in *Manduca sexta*. Nodulation is the aggregation of haemocytes in response to infection. These aggregations then become melanised forming a nodule that quarantines it. This infection response is similar to encapsulation seen in *Drosophila*. This was shown by injection of bacteria into the haemocoel of *Manduca sexta* causing nodulation. Injection of dexamethasone (a PLA₂ inhibitor) reduced the ability to clear the infection by inhibiting nodule formation. Nodulation was recovered by co-injection with either AA or EPA (Miller et al., 1994). The effects of eicosanoids in mediating nodulation have been shown in the beet armyworm, *Spodoptera exigua* and *Rhodnius prolixus* (Garcia et al., 2004; Shrestha and Kim, 2008). Collectively, these studies suggest that eicosanoids play an early role in helping to clear infections. Without them, this process is compromised.

There is contradicting evidence for the presence of eicosanoids and their precursor AA in *Drosophila*. Initial experiments, showed that homogenates were able to synthesise a range of HETE acids when incubated with exogenous AA (a product of lipoxygenase processing of AA) and were detected using gas chromatography-mass spectrometry (GC-MS) (Pagés et al., 1986). However, endogenous AA could not be detected in homogenates, even though considerable amounts of linoleic acid were detected (the precursor of AA).

The presence of AA has also been profiled in *Drosophila* heads, given the important role phosphatidylinositol (PI) plays in photoreceptor signalling. These studies failed to detect AA, and the precursors γ -linolenic (18:3) and dihomo γ -linolenic acid (20:3) (Yoshioka et al., 1985). This has been confirmed by recent LC-MS studies. *Drosophila* fed on a base diet and a diet rich in PUFA precursors were analysed and shown to lack AA suggesting that *Drosophila* cannot synthesise AA *in vivo*. These

studies also confirmed using BLAST, the absence of $\Delta 6$ and $\Delta 5$ desaturases involved in the synthesis of AA (Shen et al., 2010). Although it was shown that AA couldn't be endogenously synthesised, AA could be detected when flies were fed on a diet supplemented with AA (20:4 n-6). It is possible that *Drosophila* are like cats, being unable to synthesise AA and being dependent on acquiring AA through the diet (Bauer, 2007), but possessing the necessary machinery to process AA to prostaglandins. Indeed, radioimmunoassay using anti-prostaglandin E (PGE), indicated that PGE₂ was present within these extracts (Pagés et al., 1986). This raised the possibility that eicosanoid generating pathways were present in *Drosophila*.

Consistent with this, functional studies suggest a role for prostaglandins in ovarian follicle maturation in *Drosophila* (Tootle and Spradling, 2008). Follicle maturation was dramatically reduced when treated with COX inhibitors such as aspirin. However, maturation could be rescued when ovaries were treated with an exogenous stable form of prostaglandin. A *Pxt* (*Peroxinectin-like*, a *Drosophila* peroxidase) was proposed as a COX candidate, having homology to COX. Knock out of the *Pxt* reduced ovarian follicle maturation (Tootle and Spradling, 2008). The same group, elucidated the follicle maturation phenotype seen in the absence of prostaglandin production is due to defects in actin remodelling by the *Drosophila Singed*, a Fascin homologue. Fascin in mammals is known to interact with prostaglandins (Groen et al., 2012). These new findings provide an interesting insight into the role prostaglandins may play in *Drosophila*. Though there has been no clarification of endogenous prostaglandins in *Drosophila* and immune function of prostaglandins. To explore potential links between lipid mediators and inflammation in *Drosophila*, we exploited a sensitised background that over-activates the JAK/STAT pathway, leading to an inflammatory phenotype.

1.3 Gain-of-function *Drosophila* JAK/STAT mutation as a model for inflammation

The JAK/STAT pathway was first identified in mammals. In the canonical JAK/STAT signalling pathway ligand binding causes dimerisation of a receptor, bringing together two JAKs, which trans-phosphorylate each other eliciting the recruitment of the STAT. These are then phosphorylated, dimerise and translocate into the nucleus to bind to STAT induced transcriptional targets (Rawlings et al., 2004). The JAK/STAT pathway is evolutionary conserved and present with *Drosophila*. While mammalian JAK/STAT signalling can be mediated by multiple cytokines and growth factors, *Drosophila* only have three ligands, Unpaired (Upd), Upd2, Upd3, of which Upd is the most potent (Wright et al., 2011). Upd binds to the receptor Domeless, leading to trans-phosphorylation of Hopscotch (the *Drosophila* homologue of human Janus Kinase 3 (JAK3)) causing recruitment of Stat92E the *Drosophila* homologue of STAT5a, and the sole *Drosophila* STAT.

A gain-of-function mutation in the *hopscotch* gene, called *Tumourous-lethal* (*hop*^{Tum}), leads to an inflammatory melanotic tumour phenotype. The *hop*^{Tum} mutation is caused by a single amino acid G341E substitution (Harrison et al., 1995). The *hop*^{Tum-l} mutation increases the level of tyrosine phosphorylation of the mutant JAK compared to the wild type. Thus the *hop*^{Tum} mutation causes constitutive, ligand independent activation of the JAK/STAT pathway (Harrison et al., 1995) (Figure 5). This mutation is temperature sensitive conditional. This means that the phenotype is more severe at elevated temperatures. For example, at temperatures above 25°C, the induction of melanotic tumours is severe, but the tumour phenotype is milder at lower temperatures (Luo et al., 1995). Melanotic tumours are aggregations of haemocytes that are encapsulated in a melanised capsule (Barigozzi, 1958)

Like mammalian JAKs, *Drosophila* JAK is involved in the regulation of haematopoiesis. Studies have shown that the *hop*^{Tum} mutation causes the lymph gland to become hypertrophic due to increased proliferation of plasmatocytes (Luo et al., 1995). These plasmatocytes have also been shown to prematurely differentiate into lamellocytes. The increase in cell numbers is speculated to cause aggregation of haemocytes forming clumps, resulting in the production of melanotic tumours. Crystal cells appear to be unaffected by the mutation (Silvers and Hanratty, 1984).

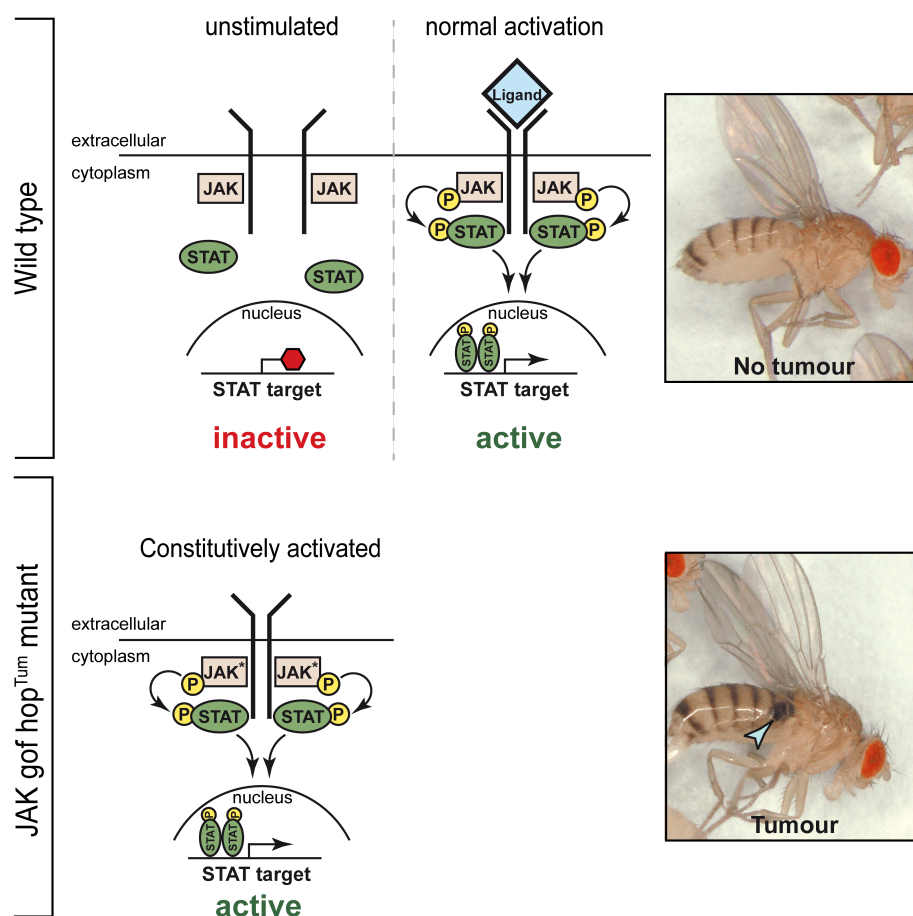


Figure 5. JAK gain-of-function *hop*^{Tum} signalling

Wild type JAK/STAT signalling is ligand-dependent. In the presence of a ligand, transphosphorylation of JAKs occurs, resulting in translocation of STATs to the nucleus. In the *hop*^{Tum} mutant, signalling is constitutive and ligand-independent. The constant signalling of JAK/STAT results in a melanotic tumour phenotype that can be visualised as a black mass in the fly (arrow). Figure adapted from Kwon et al., 2008

This *hop*^{Tum} mutant provides an extremely useful model for chronic inflammation. Most inflammatory models in use rely on a mutant background that initiates a phenotype that can be used to disentangle mechanisms that regulate the phenotype. Such models include the apolipoprotein deficient mouse that is used to study atherosclerosis and the collagen-induced arthritis mouse model. I have used the *hop*^{Tum} mutant in a similar way to initiate an inflammatory response, by overstimulating the JAK/STAT pathway resulting in the formation of an inflammatory melanotic tumour. This phenotype can aid in identifying downstream mediators of inflammation that are required to sustain inflammation i.e. the signals that orchestrate the haemocytes to aggregate forming the melanotic tumour.

1.4 Aims and objectives

Inflammatory disease is on the rise due to a combination of lifestyle, environment and genetic predisposition. Inflammatory diseases have extensive effects on health and economic costs. For example, cardiovascular disease (CVD) alone, is responsible for 40% of all deaths in the EU and costs the EU economy approximately €196 million per year (European Society of Cardiology). There is a requirement for new therapeutic targets to be developed and *in vivo* models that can be used to dissect underlying inflammatory mechanisms. We believe *Drosophila* could be a useful tool in aiding inflammatory research.

The main aim of this study was to characterise *Drosophila* inflammatory lipid mediators and precursors. Given conflicting evidence, regarding presence of AA and prostaglandins, we also wanted to clarify whether *Drosophila* can synthesise AA and subsequently produce prostaglandins. To do this we used an inflammatory model based on the gain-of-function temperature sensitive *hop*^{Tum} mutant, which results in an inflammatory phenotype, a melanised inflammatory mass. This model allowed us to screen for effectors of inflammation.

The **first** objective was to identify factors, which could increase or reduce the inflammatory phenotype of our model. This involved dietary supplementation of essential fatty acids, the use of COX and MPO inhibitors, genetic knockdown of putative COX/MPO *Drosophila* homologues and known genes involved in lipid liberating pathways. The **second** objective was to biochemically analyse *Drosophila* samples, using LC-MS to identify directly if endogenous lipid mediators were present, and if fatty acid supplementation affected the presence of them. **Thirdly**, we attempted to clarify the actions of the lipid mediators identified by LC-MS by treating S2 cells and analysing gene expression. **Fourthly**, characterised the enzymatic actions *in vitro* of the *Drosophila* COX/MPO putative homologues identified using recombinant protein. And characterised the *in vivo* actions of the *Drosophila* COX/MPO putative homologues using sterile and septic assays.

2. Material and Methods

2.1 *Drosophila* husbandry

Drosophila were raised on wheat flour, yeast agar media of 10 ml per vial. Media was prepared using 550 g dextrose, 500 g yeast, 350 g flour and 80 g of agar (Drewitt) sequentially added to 11 L of water and then autoclaved. Once cooled, 25 g of Nipagin (MP Biomedicals, LLC) was dissolved in 250 ml of ethanol and added along with 40 ml of propionic acid (Sigma). Media was decanted, left to cool, capped, and stored in a cold room.

Drosophila were incubated at 25°C unless otherwise stated, and transferred to fresh vials weekly.

2.2 *Drosophila* inflammatory tumour assays

The *hop*^{Tum} strain carries a gain-of-function, temperature sensitive mutation in the *Drosophila* JAK (Luo et al., 1995a) that causes the initial inflammatory insult. *FM7* is a balancer chromosome (Merriam, 1969) that prevents recombination, and allows selection of the *hop*^{Tum} mutation. *hop*^{Tum}/*FM7* virgin females were harvested from a stock maintained by crossing *hop*^{Tum}/*FM7* females with *FM7*/*Y* males. This stock was incubated at 25°C and was perpetuated by transferring to fresh vials every two days.

For inflammatory tumour assays five *hop*^{Tum}/*FM7* females were crossed with either five *w*¹¹¹⁸ males for the control/reference point for all experiments, or five mutant males (in the case of mutant enhancer/suppressor studies) per vial. Crosses were allowed to mate for two days before being transferred to fresh media. Five initial vials were set up for each datum point and were incubated for 3 days before being transferred to a second fresh set of vials, giving a total of 10 replicates per experiment. Crosses were incubated for 3 days and the adult flies then removed. The resultant *hop*^{Tum} females were scored for both tumour incidence and severity. All

crosses were maintained on standard media or supplemented media where stated and all crosses were incubated at 27°C unless otherwise stated.

Tumour incidence and severity was scored using a Nikon SMZ645 stereomicroscope equipped with a C-FM reticule. The reticule has 0 to 100 notches, with each notch equal to 20 μm at full magnification. Tumours were measured using the highest magnification. Seven tumour size categories were used according to notches on the reticule: 0, 0.1-2.5, 2.6-5, 5.1-7.5, 7.6-10, 10.1-15 and 15+ which equates to: 0, 2-50, 52-100, 102-150, 152-200, 202-300, and 300+ μm .

Tumour incidence percentage for each replicate was calculated by dividing the number of tumour containing females by the total number of females and multiplied by 100. The mean and standard deviation of ten vials for each experimental regime was calculated. T-tests were used to determine statistical significance. A P-value threshold of 0.001 was used. Microsoft Office Excel 2010 was used to input data and generate calculations. The programme R was used to generate box and whisker plots. The upper line to the box represents upper quartile, the middle line represents the mean, and the lower line of the box is the lower quartile value. The upper line of the whisker is the greatest value, and the lower whisker is the lowest value. Any circles above and below the whiskers are outliers. Bubble graphs were generated from the programme Many Eyes (<http://www-958.ibm.com>) using the matrix chart function to depict tumour severity categories.

2.2.1 Dietary supplementation of fatty acids

Unrefined organic cold pressed safflower oil (Clear spring), which contains 64% linoleic acid (an omega-6 unsaturated fatty acid), was supplemented to media spanning five different concentrations. These were: 0%, 0.1%, 0.3%, and 0.7%.

Organic cold pressed flaxseed oil (Food Supplement Company), which contains 55% of α -linolenic acid (an omega 3 unsaturated fatty acid), was supplemented to media at the following concentrations: 0%, 0.1%, 0.3% and 0.7%. Flaxseed oil was also added in conjunction with safflower oil. Safflower oil was added at a constant concentration of 0.15% while simultaneously increasing flaxseed concentrations from 0%, 0.05%, 0.1% and 0.15%.

Coconut oil (Sigma) containing 85% saturated fat was added to media at the following concentrations: 0%, 0.1%, 0.3%, 0.7% and 1%. Cholesterol (Sigma) was dissolved in ethanol to make a stock solution and then aliquoted to media to make the following concentrations: 0%, 0.001%, 0.005%, 0.01% and 0.05%.

For all supplementation experiments, *hop*^{Tum}/*w*¹¹¹⁸ female progeny from a cross between five *hop*^{Tum}/*FM7* females and five *w*¹¹¹⁸ males were analysed from each vial. These experiments were carried out at both 25°C and 27°C. The same experimental protocol was used as is outlined in section 2.2.

2.2.2 MPO and COX Inhibitors

A range of concentrations was used for each MPO and COX inhibitor, which spanned the published IC50 values. Inhibitors were dissolved in dimethyl sulfoxide (DMSO, Sigma) and then added to fly media before pouring. The same screen protocol was used as outlined earlier in section 2.2. All COX inhibitors were obtained from Sigma; MPO Inhibitor 1 was obtained from Calbiochem.

Aspirin was supplemented at the following concentrations: 0 mM, 0.3 mM, 0.7 mM, 1 mM, 3 mM, 7 mM, 10 mM and 30 mM. These spanned the published ovine IC50 of 0.75 mM for COX-1 and 1.25 mM for COX-2 (Johnson, Wimsatt, Buckel, Dyer, & Maddipati, 1995).

NS-398 was supplemented at the following concentrations: 0 μ M, 0.3 μ M, 0.7 μ M, 1 μ M, 3 μ M, 7 μ M, 10 μ M and 30 μ M. These spanned the published human recombinant COX IC₅₀ of 75 μ M for COX-1 and 1.77 μ M for COX-2 (Barnett et al., 1994).

SC-560 was supplemented at the following concentrations: 0 nM, 7 nM, 10 nM, 30 nM, 70 nM, 100 nM, 300 nM, 700 nM, 1 μ M, 3 μ M, 7 μ M, and 10 μ M. These spanned the published human recombinant COX IC₅₀ of 9 nM for COX-1 and 6.3 μ M for COX-2 (Smith et al., 1998).

MPO Inhibitor 1 was supplemented at the following concentrations: 0 nM, 10 nM, 30 nM, 50 nM, 100 nM, 200 nM, 300 nM, and 1000 nM. These spanned the published purified MPO IC₅₀ of 300 nM (Calbiochem).

2.2.3 Putative *Drosophila* MPO/COX homologues

BLAST (Altschul et al., 1990) was used to identify *Drosophila* homologues of human COX-1, COX-2 and MPO based on amino acid sequence similarity. Mutant strains of each potential homologue were used. Mutant strains were either due to a point mutation, an insertion or a deficiency depending on availability.

Five males from each mutant line were crossed with five *hop*^{Tum}/FM7 females. Females from the progeny that contained both the *hop*^{Tum} and homologue mutation were collected and scored for tumour incidence. The tumour assay is detailed in section 2.2. Details of mutants used are outlined in Table 1.

Table 1. Mutant strains of MPO/COX homologues

| Genotype | Description |
|---|---|
| <i>w¹¹¹⁸; Df(3R)ED5785, P{3'.RS5+3.3'}ED5785/TM6C, cu¹ Sb¹</i> | A chromosomal deletion of 3R:13,543,832-13,769,792. This removes <i>Peroxinectin-like (Pxt)</i> mapped to 3R:13,545,176-13,548,677 [+]. |
| <i>y¹ w^{67c23}; P{EPgy2}^{PxtEY03052}</i> | A transposable element insertion that is located at sequence: 3R:13,545,179 [+]. This affects <i>Pxt</i> . |
| <i>PBac{WH}Pxt^{U5258}/TM6b, Tb¹, Hu¹</i> | A transposable element insertion that is located at sequence: 3R:13,546,640 [+]. This affects <i>Pxt</i> . |
| <i>w¹¹¹⁸; Df(3L)BSC119/TM6B, Tb¹</i> | A chromosomal deletion of 3L:2,600,282-2,823,614. This affects <i>Peroxidasin (Pxn)</i> mapped to 3L:2,601,455-2,630,072 [-]. |
| <i>w¹¹¹⁸; Df(3L)Exel6091, P{XP-U}Exel6091/TM6B, Tb¹</i> | A chromosomal deletion that deletes segment 3L:2,656,263-2,821,245. It was used in an overlap assay as it removes genes <i>Df(3L)BSC119</i> does, but not <i>Pxn</i> itself. |
| <i>w¹¹¹⁸; Mi{ET1}Irc^{MB11278}</i> | A transposable element insertion located at sequence 3R:12,829,982. This affects gene <i>Immune regulated catalase (Irc)</i> mapped to 3R:12,828,597-12,832,930. |
| <i>w¹¹¹⁸; Df(3R)BSC792, P+PBac{XP3.WH3}BSC792/TM6C, Sb¹ cu¹</i> | A chromosomal deletion of 3R:12,808,916-12,890,724. This affects gene <i>Irc</i> . |
| <i>w¹¹¹⁸; Df(3R)Exel8165, repo⁸¹⁶⁵/TM6B, Tb¹ Tb¹</i> | A chromosomal deletion of 3R:12,838,655-12,879,706. This affects <i>Peroxidase (Pxd)</i> , mapped to 3R:12,840,298-12,853,709 [+], and <i>cardinal (cd)</i> , mapped to 3R:18,520,681-18,523,712 [+]. |
| <i>w¹¹¹⁸; Df(3R)Exel6192, P{XP-U}Exel6192/TM6B, Tb¹</i> | A chromosomal deletion of 3R:18,492,372-18,724,923. This affects <i>cd</i> . |
| <i>w¹¹¹⁸; Df(2L)BSC149/CyO</i> | A chromosomal deletion of 2L:18,444,727-18,673,286. This affects <i>CG10211</i> mapped to 2L:18,517,487-18,531,967 [+]. |
| <i>w⁺; P{FRT(w^{ns})}2A P{neoFRT}82B PBac{SAstopDsRed}LL00313 P{Car20y}96E / TM3, Sb¹</i> | A transposable element insertion located at sequence 3R:12,850,518. This affects gene <i>Pxd</i> mapped to 3R:12,840,298-12,853,709 [+]. |
| <i>Df(2L)C144, dpp^{d-no} ed¹/In(2LR)Gla, wg^{Gla-1} Bc¹ Egfr^{E1}</i> | chromosomal deletion of 2L:2,517,598-2,955,279. This affects gene <i>Dual oxidase (dDuox)</i> mapped to 2L:2,815,970-2,830,248. |
| <i>w¹¹¹⁸; Df(2L)BSC692, P+PBac{XP3.RB5}BSC692/SM6a</i> | A chromosomal deletion of 2L:2,830,265-2,868,633. This affects gene <i>dDuox</i> . |

2.2.4 RT-PCR verification of MPO/COX homologue insertions

To confirm reduced expression in insertion strains five male flies from *Pxt*^{f05258}, *Irc*^{MB11278} and *w*¹¹¹⁸ (control) were collected and snap-frozen in liquid nitrogen. A μ MACS mRNA isolation kit (Miltenyi Biotec Ltd.Uk) was used to isolate mRNA. The kit uses Oligo (dT) MicroBeads that hybridise to the polyA tail of mRNA. This magnetically labels the mRNA allowing isolation when a μ Column is placed in a MACS separator. mRNA isolation was carried out according to the manufacturer's protocol. Eluted mRNA was precipitated with 90 μ l isopropanol (AnalaR NORMAPUR), 9 μ l 3 M NaOAc (Sigma), and 1 μ l glycogen (Sigma), and incubated at -80°C for 15 min. Samples were centrifuged for 15 min at 21, 000 *g* and the supernatant removed. Pellets were reconstituted in 150 μ l 70% of ethanol (AnalaR NORMAPUR) and centrifuged again as before but for 5 min. The supernatant was removed and pellets left to air-dry before being re-suspended in 10 μ l nuclease-free deionised water (HyClone).

cDNA was generated using a 50 μ l reaction mix consisting of: 1x First Strand Buffer, 1 mM DTT, 0.5 mM dNTP, 2 units of RNaseOUT, 4 units of Superscript II reverse transcriptase, 0.1 mM Oligo(dT), and 10 μ l mRNA. The reaction was carried out in a Peltier Thermal Cycler-200 (MJ Research) using a program of: 50°C for 1 hour and 70°C for 15 min continuing at 4°C. cDNA was stored at -20°C. All cDNA reaction reagents were from Invitrogen.

RT-PCR analysis was carried out using a StepOnePlus RT-PCR system (Applied Biosystems). 10 μ l reactions consisted of: 1x Absolute SyBR Green ROX mix (THERMO scientific), 200 ng cDNA, 10 pmol 5' primer and 10 pmol 3' primer. The RT-PCR program started with 95°C for 10 min as a hot-start enzyme was used, and proceeded with 40 cycles of 95°C for 15 sec and 60°C for 1 min. A melt curve was

also generated. C_T values were used for analysis. *RpL32* was the endogenous control used for normalisation. Primers used are shown in table 2.

Table 2. RT-PCR primers used to confirm *Pxn* and *Irc* levels in mutant strains

| Gene | Orientation | Primer sequences (5' to 3') |
|--------------|--------------------|------------------------------------|
| <i>RpL32</i> | 5' | CAACAGAGTCGGTCGCCGCTTCAACGGACA |
| | 3' | CAGCTCGCGCACGTTGTGCACCAGGAACTT |
| <i>Pxt</i> | 5' | GCAGCTCCTCGATGTGATTGAAAC |
| | 3' | CTAGAGTGCGAGCGAGAGGTAAGA |
| <i>Irc</i> | 5' | TGATCTACACGATCTTCATGC |
| | 3' | CTCCTCAATGACCACTCGACG |

2.2.5 Putative *Drosophila* free fatty acid liberating/signalling pathway homologues

BLAST (Altschul et al., 1990) was used to identify *Drosophila* homologues of pathway components that lead to the production of free fatty acids, or are involved in lipid signalling. Strains used to remove putative pathway homologues are outlined in Table 3. The same tumour assay as detailed in section 2.2 was used.

Table 3. Fatty acid pathway component strains

| Genotype | Description |
|---|--|
| <i>w[*] norpA³³</i> | An ethyl methanesulfonate (EMS) mutation located at X:4,251,391 caused by a nucleotide substitution C1370T, that results in a premature termination codon. This affects <i>no receptor potential A (norpA)</i> located at X:4,216,659-4,259,417 [+], a homologue of Phospholipase C (PLC). |
| <i>w[*] norpA³⁵</i> | An EMS mutation located at X:4,251,818, which results in an amino acid substitution C361T. This affects <i>norpA</i> . |
| <i>w[*] norpA³⁶</i> | A loss of function EMS mutation located at X:4,253,449. It is a 28bp deletion that causes a frame shift, deleting 9 amino acids. This affects <i>norpA</i> . |
| <i>norpA⁴⁵</i> | An unmapped mutation in <i>norpA</i> . |
| <i>y¹ w^{67c23}.P{SUPorP} Plc21C^{KG05991}</i> | A transposable element insertion in <i>Phospholipase at 21C (Plc21C)</i> located at 2L:310,245 [+]. This affects <i>Phospholipase at 21C (Plc21C)</i> located at 2L:305,935-347,927 [+], which is a homologue of PLC. |
| <i>y¹ w^{67c23}.P{SUPor-P} Plc21C^{KG08451}</i> | A transposable element insertion in <i>Plc21C</i> that is located at 2L:306,162 [-]. |
| <i>rud¹ v^{54k} tc¹ sl² smd¹/FM7c, sn[*]</i> | An X-ray induced loss of function allele in <i>small wing (sl)</i> caused by a 13 base pair deletion located at X:16,246,728. <i>small wing (sl)</i> is located at X:16,244,348-16,250,515 [+] and is a homologue of PLC. |
| <i>w¹¹¹⁸; Df(2R)BSC263/ CyO</i> | A chromosomal deletion of 2R:3,034,369-3,334,915. This affects <i>secretory Phospholipase A2 (sPLA2)</i> located at 2R:3,330,369-3,331,332 [+]. |
| <i>w¹¹¹⁸; Df(2R)BSC264/ CyO</i> | A chromosomal deletion of 2R:3,283,390-3,377,339 that includes <i>sPLA2</i> . |
| <i>Gα49B¹</i> | An EMS mutation that is a hypomorphic allele at location 2R:8,504,576. This affects <i>G protein α q subunit (Gαq)</i> located at 2R:8,500,245-8,510,702 [+], a homologue of Gαq. |

| | |
|---|--|
| <i>sktl</i> ^{Δ15} | A loss of function allele caused by an imprecise excision of the P element (<i>fam</i> ⁷⁵⁰⁵) located in an intron of <i>skittles</i> (<i>sktl</i>). <i>Sktl</i> is located at 2R:16,714,787-16,719,923 [-] and is a homologue of phosphatidylinositol 4-phosphate (PI4P). |
| <i>sktl</i> ^{Δ20} | An imprecise excision of the P element (<i>fam</i> ⁷⁵⁰⁵) located in an intron of <i>sktl</i> , resulting in a loss of function allele. |
| <i>inaE</i> ^{xl15} | An imprecise excision of the progenitor insertion <i>P{SUPor-P}inaE</i> ^{KG08585} . This affects <i>inactivation no afterpotential E</i> (<i>inaE</i>) located at X:13,677,747-13,705,526 [+]. A homologue of diacylglycerol lipase (DAG lipase). |
| <i>inaE</i> ^{xl18} | An imprecise excision of the progenitor insertion in <i>inaE P{SUPor-P}inaE</i> ^{KG08585} . |
| <i>rdgA</i> ¹ | An EMS mutation located at X:8,915,781 causing a nucleotide substitution, C3695T in <i>retinal degeneration A</i> (<i>rdgA</i>) located at X:8,801,101-8,923,509 [+]. This is a homologue of diacylglycerol kinase (DGK). |
| <i>ltp-r83A</i> ^{sv35} | An EMS mutation in <i>Inositol 1,4,5,-tris-phosphate receptor</i> (<i>ltp-r83A</i>) located at 3R:1,360,239 that causes an amino acid change Q1572. It is a homologue of Inositol 1, 4, 5-triphosphate receptor. |
| <i>ltp-r83A</i> ^{ka1091} | An EMS mutation in <i>ltp-r83A</i> located at 3R:1,361,196 this causes an amino acid substitution G1891S. |
| <i>ltp-r83A</i> ^{ka901} | An EMS mutation in <i>ltp-r83A</i> located at 3R:1,363,712 resulting in an amino acid substitution G2630S. |
| <i>y</i> ¹ <i>w</i> ^{67c23} , <i>P{EPgy2}</i> ^{EY04862} | A transposable element insertion located at 2R:16,758,286 [+]. This affects the gene <i>lpk1</i> located at 2R:16,758,296-16,766,650 [+], a homologue of inositol polyphosphate kinase 1 (IPK1). |
| <i>w</i> ¹¹¹⁸ ; <i>Df(2R)BSC404/CyO</i> | A chromosomal deletion of 2R:16,554,779-16,770,204 that includes <i>lpk1</i> . |
| <i>w</i> ¹¹¹⁸ ; <i>Df(2R)Exel6071, P{XP-U}Exel6071/CyO</i> | A chromosomal deletion of 2R:16,723,538-16,944,303 that includes <i>lpk1</i> . |
| <i>y</i> ¹ <i>w</i> ^{67c23} , <i>P{GSV6}GS15129/SM1</i> | A transposable element insertion located at 2L:574,230, affecting <i>lpk2</i> located at 2L:573,033-574,264 [-], a homologue of inositol polyphosphate kinase 2 (IPK2). |
| <i>y</i> ¹ <i>w</i> ^{67c23} , <i>P{GSV6}GS14317/SM1</i> | A transposable element insertion located at 2L:574,219, affecting <i>lpk2</i> located at 2L:573,033-574,264 [-], a homologue of IPK2. |
| <i>w</i> ¹¹¹⁸ ; <i>Df(2L)ED49, P{3'.RS5+3.3}ED49/SM6a</i> | A chromosomal of 2L:568,095-587,983 that includes <i>lpk2</i> . |
| <i>w</i> ¹¹¹⁸ , <i>Df(2R)BSC382/CyO</i> | A chromosomal deletion of 2R:12,663,669-12,789,130, affecting <i>inactivation no afterpotential C</i> (<i>inaC</i>) located at 2R:12,786,040-12,789,080 [+]. A homologue of protein kinase C (PKCα/β). |
| <i>w</i> ¹¹¹⁸ ; <i>Df(3R)Exel621, P{XP-U}Exel6211/TM6B, Tb¹</i> | A chromosomal deletion of 3R:24,816,740-24,889,986 that includes Protein C kinase 98E (<i>Pkc98E</i>) located at 3R:24,864,878-24,874,870 [+], that is a homologue of PKCε. |
| <i>w</i> ¹¹¹⁸ ; <i>Df(3R)BSC806, P+PBac{XP3.RB5}BSC806/TM6C, Sb¹ cu¹</i> | A chromosomal deletion of 3R:24,696,033-24,938,249 that includes <i>Pkc98E</i> . |

| | |
|---|---|
| <i>y* w*</i> ; <i>P{neoFRT}40A</i> <i>P{FRT(whs)}G13 cn1</i> <i>PBac{SAstopDsRed}LL</i> <i>04932 bw¹/CyO, S* bw¹</i> | A transposable element insertion located at 2R:12,813,988-12,813,988 [-] that affects <i>Protein C kinase 53E (Pkc53E)</i> located at 2R:12,812,160-12,832,631 [+]. A homologue of PKC α/β . |
| <i>y¹ w^{67c23},</i> <i>P{EPgy2}^{Pkc53EEY14093}/</i> <i>CyO</i> | A transposable element insertion in <i>Pkc53E</i> located at 2R:12,812,005-12,812,005 [+]. |
| <i>w¹¹¹⁸; Df(2R)ED2751,</i> <i>P{3'.RS5+3.3}ED2751/</i> <i>SM6a</i> | A chromosomal deletion of 2R:12,744,676-12,984,808 that includes <i>Pkc53E</i> . |
| <i>y¹ w^{67c23},</i> <i>P{lacW}aPKC^{k06403}/CyO</i> | A transposable element insertion located at 2R:10,840,608-10,840,608 [-] affecting <i>atypical protein kinase C (aPKC)</i> located at 2R:10,831,963-10,850,474 [-]. |
| <i>y* w*</i> ; <i>P{neoFRT}40A</i> <i>P{FRT(whs)}G13 cn1</i> <i>PBac{SAstopDsRed}LL</i> <i>03594 bw¹/CyO, S* bw¹</i> | A transposable element insertion in <i>aPKC</i> located at 2R:10,846,147-10,846,147 [-]. |
| <i>Df(1)BSC543,</i> <i>w¹¹¹⁸/FM7h/Dp(2;Y)G,</i> <i>P{hs-hid}Y</i> | A chromosomal deletion of X:11,815,319-12,352,077 that includes <i>protein kinase C δ (PKCδ)</i> located at X:12,326,25712,347,683 [+]. |
| <i>Df(1)ED7153, w¹¹¹⁸</i> <i>P{3'.RS5+3.3}ED7153/</i> <i>FM7h</i> | A chromosomal deletion of X:11,901,120-2,461,493 that includes <i>PKCδ</i> . |
| <i>Df(1)BSC623,</i> <i>w¹¹¹⁸/Binsincy</i> | A chromosomal deletion of X:12,289,725-13,025,219 that includes <i>PKCδ</i> . |
| <i>Eip75B^{Δ1}/TM6B, P{Ubi-</i> <i>GFP.S65T}PAD2, Tb¹</i> | A 3kb deletion by imprecise excision of <i>P{PZ}Eip75B⁰³³³⁸</i> affecting <i>Ecdysone-induced protein 75B (Eip75B)</i> located at 3L:17,944,053-18,057,796 [-]. A homologue of PPAR γ . |
| <i>Eip75B^{Δ51}/TM6B, P{Ubi-</i> <i>GFP.S65T}PAD2, Tb¹</i> | A 30kb deletion by imprecise excision of <i>P{PZ}Eip75B⁰³³³⁸</i> affecting <i>Eip75B</i> . |
| <i>w¹¹¹⁸; Df(3L)Exel6133,</i> <i>P{XP-</i> <i>U}Exel6133/TM6B, Tb¹</i> | A chromosomal deletion of 3L:18,009,803-18,132,421 that includes <i>Eip75B</i> . |
| <i>w¹¹¹⁸; Df(3L)ED224,</i> <i>P{3'.RS5+3.3}ED224/T</i> <i>M6C, cu¹ Sb¹</i> | A chromosomal deletion of 3L:17,962,303-18,391,619 that includes <i>Eip75B</i> . |
| <i>w¹¹¹⁸,</i> <i>Df(3L)BSC775/TM6C,</i> <i>Sb¹ cu¹</i> | A chromosomal deletion of 3L:17,788,244-18,891,426 that includes <i>Eip75B</i> . |

2.3 Lipidomics and functional characterisation of fatty acids

hop^{Tum}/FM7 females were crossed with *w*¹¹¹⁸ males and reared on standard media, 0.15% Omega-3 supplemented media and 0.15% Omega-6 supplemented media and incubated at 27°C. Wandering third instar *hop*^{Tum}/FM7 and *w*¹¹¹⁸ (control) larvae were harvested, washed with water, and weighed to make 100 mg and 200 mg samples. These were snap-frozen in liquid nitrogen and stored at -80°C. Samples were sent to our collaborators, Professor Anna Nicolaou and Karen Massey for liquid chromatography-mass spectrometry analysis (LC-MS). Samples were analysed for arachidonic acid, prostaglandins, hydroxy-octadecadienoic acids (HODEs) and chiral enantiomers of HODEs. Detailed methods and materials of the lipidomics can be found in Massey & Nicolaou 2013.

2.3.1 Lipid extraction

200 mg larvae samples were defrosted and transferred to a Dounce glass homogeniser. Samples were homogenised in 500 µl ice-cold methanol using a glass pestle and mortar. The homogenate was transferred to a glass vial. The mortar was washed twice with 100 µl methanol and added to the homogenate. Ice cold water was added to make a final 15% v/v methanol:water homogenate. 20 µl of 1 ng/µl internal standard PGB₂-d₄ (Cayman Chemicals, Ann Arbor, MI) was added. The homogenate was left for 15 min on ice, in the dark and then centrifuged for 10 min, at 4°C, at 130 g to remove precipitated proteins. The supernatant was transferred to a glass vial and acidified to pH 3.0 using 0.1 M HCL. Acidified extracts were applied to the pre-conditioned solid phase extraction (SPE) cartridges (C18-E, Phenomenex, Macclesfield, UK) where lipid mediators were eluted with methyl formate. Extracts were dried under nitrogen, in the dark and reconstituted in 100 µl 70/30:v/v ethanol/water.

2.3.2 LC/ESI-MS/MS analysis

Lipid mediator analysis was carried out using a Waters Alliance 2695 HPLC pump coupled to an ESI triple quadrupole Quattro Ultima mass spectrometer (Waters, Elstree, Hertsfordshire, UK). For whole lipid analysis and identification of prostaglandins PGE₂ and PGF_{2α}, as well as 9- and 13-HODE, chromatographic separation was carried out using a C18 Luna 5 µm, 150 x 2.0 mm column (Phenomenex). For identification of 9- and 13-HODE chiral enantiomers, chromatographic separation was carried out using a LUX cellulose-1 3 µm, 150 x 2.0 mm column (Phenomenex).

Analytes were analysed using MassLynx version 4.0 via multiple reaction monitoring (MRM) mode to identify lipid mediators of interest using their known MRM transitions from the standards used. PGE₂ MRM transitions used: m/z 351>271, m/z 351>315, m/z 351>333. PGF_{2α} MRM transitions used: m/z 353>193, m/z 353>247, m/z 353>309. MRM transitions for 9-HODE: 295>171 and 13-HODE: 295>195. Relative abundance of lipid mediators was calculated from the area underneath the peak of detection seen in the LC-MS plots.

2.3.3 Fat body prep and Oil Red O staining

w¹¹¹⁸ flies (control) and *hop^{Tum}/FM7* females crossed with *w¹¹¹⁸* males were incubated at 29°C. Wandering third instar *hop^{Tum}/FM7* and *w¹¹¹⁸* (control) progeny larvae were collected and washed with water. 5-10 Larvae were ripped open in 200 µl of HyQ-CCM3 (HyClone) containing 1x complete mini protease inhibitors (Roche) on ice. Larvae were held at the mouth hook with fine forceps and teared open using another pair of forceps to the posterior end. Haemocytes pour out into the media when the larvae are teared and these were used for Oil red O staining in section 2.3.4. The carcasses were placed in 1 ml of 1xPBS containing 3.7% formaldehyde (Sigma), and

fixed for ten minutes on ice. Carcasses were then carefully washed 5 times with 1xPBS. 1 ml of Oil Red O working solution (600 μ l 0.1% Oil Red O (Sigma) and 400 μ l water) was added for 1 min, and then removed. 1 ml of isopropanol was used to wash, pipetted up and down and removed immediately. Fat bodies were washed with 1xPBS and the fat body was dissected from the carcass using a microscope and placed in 200 μ l 1xPBS. Fat body dissections were centrifuged onto glass slides for 5 min at 28 g on low acceleration and deceleration settings, using a cytospin3 (Shandon). Centrifuged fat bodies were fixed with 100 μ l 4% paraformaldehyde for 10 min, and washed with 1xPBS for 10 min and mounted using Vectashield containing DAPI (Vecta Laboratories). Slides were visualised using a Zeiss Axiovert 100M confocal microscope.

2.3.4 Haemocyte Oil Red O staining

Haemocytes generated during the fat body preparations were centrifuged at 400 g for 3 min at 4°C to pellet the haemocytes. The supernatant was removed and cells re-suspended in 50 μ l of HyQ-CCM3 and pipetted onto a Multispot slide (PH-001; C. A. Hendley) and incubated for 30 min at room temperature. The media was removed and cells were fixed with 3.7% paraformaldehyde for 10 min and then washed in 1xPBS for 10 min. 50 μ l of Oil Red O working solution was added and incubated for 2.5 min. The slide was then de-stained using isopropanol and washed with 1xPBS three times for 3 min and then mounted in Vectashield containing DAPI. Haemocytes were visualised using a Zeiss Axiovert 100M confocal microscope.

2.3.5 *Eip75b* ligand binding domain with GFP reporter

w¹¹¹⁸; *P{hs-GAL4-Eip75B.LBD.HA}65.1*; *P{UAS-Stinger}3* flies were incubated at 25°C for 3 days. Adult flies were removed and the vial containing progeny was heat shocked in a water bath at 38°C for 1 hour and then incubated at 25°C until progeny

reached 3rd instar wandering stage. This was to induce expression of Eip75b-LBD GAL4. Three samples of 300 larvae were ripped open in 200 µl of HyQ-CCM3 containing 1x complete mini protease inhibitors (Roche) on ice. Haemocytes were centrifuged at 400 g for 10 min at 4°C, and then washed with 1xPBS and re-suspended in either A) 250 µl HyQ-CCM3 (control) B) 250 µl HyQ-CCM3 + 20 µg/ml 9-HODE (Cayman) C) 250 µl HyQ-CCM3 + 20 µg/ml 13-HODE (Cayman). Cells were cultured in a chamber slide (Lab-Tek II) for 48 hours at 27°C. After incubation, cells were fixed with 100 µl 3.7% paraformaldehyde for 10 min. The culture chamber was removed and the slide washed with 1xPBS for 10 min. Cells were blocked overnight at 4°C in 1xPBST (1xPBS containing 0.1% Tween-20 (Sigma)) and 1% FCS. Polyclonal chicken anti-GFP antibody (Upstate Bio-technology) was added at a 1:200 dilution and incubated for 60 min. Slides were washed with 1xPBST twice for 10 min and FITC-conjugated anti-chicken IgY (H+L) (Jackson ImmunoResearch laboratories) secondary antibody added at a dilution of 1:400 and incubated for 60 min. Slides were washed twice with 1xPBST for 10 min and mounted in Vectashield containing DAPI.

2.3.6 S2 cell treatment with HODEs and Omega fatty acids for RT-PCR analysis

A 1:5 dilution of S2 cells were grown in Insect Xpress (Lonza) media for 3 days in a T25 tissue culture flask (Corning). A 1:12 dilution was made from this culture and 12 1 ml aliquots were made in 1.5 ml eppendorf tubes and centrifuged at 400 g at 4°C for 10 min. The pelleted cells were washed with 1 ml 1xPBS and re-suspended in a final volume of 250 µl. Three replicates per treatment was made: Media alone (control), media + 0.15% Omega-3, media + 0.15% Omega-6, media + 0.15% Omega-3 and -6, media + 20 µg/ml 9-HODE, and lastly media + 20 µg/ml 13-HODE. Treatments were incubated in a 24 well tissue culture plate (SARSTEDT) at 27°C for

48 hours. Cultures were then centrifuged and washed in 1xPBS and the cell pellets then stored at -80°C

mRNA was isolated, cDNA generated, and analysed for RT-PCR as described in section 2.2.4. A panel of plasmacytocyte and lamellocyte gene markers were used to analyse samples. *RpL32*, *GAPDH2* and α -*Tubulin* were used as endogenous controls. Primers used are shown in Table 4.

Table 4. RT-PCR primers used to analyse S2 treated cells

| Gene | Orientation | Primer sequences (5' to 3') | Description |
|---------------------------|-------------|---|--|
| α - <i>Tubulin</i> | 5', 3' | GTCGCGTGTGAAACACTTCCA AGCAGGCGTTTCCAATCT | <i>α-Tubulin at 84B(α-Tubulin)</i> is a housekeeping gene involved mitotic spindle formation and cytoskeleton structure. |
| <i>GAPDH2</i> | 5', 3' | CCAGAAGACCGTCGATGG TTCCGGTGAGCTTACCGTTG | <i>Glyceraldehyde 3 phosphate dehydrogenase 2 (GAPDH2)</i> is a housekeeping gene involved in glycolysis |
| <i>RpL32</i> | 5', 3' | CAACAGAGTCGGTCGCCGCTT CAACGGACA CAGCTCGCGCACGTTGTGCAC CAGGAACCT | <i>Ribosomal protein L32 (RPL32)</i> is a housekeeping gene which is involved in protein translation |
| <i>Iz</i> | 5', 3' | CTCCAACTCCATCAGCATCT CCAATCCGAGTCCGAGTCCG | <i>lozenge (Iz)</i> is a transcription factor that regulates crystal cell differentiation, therefore a marker for crystal cells. |
| <i>crq</i> | 5', 3' | GCGATCATCGAAGCGGGAAG GCATTAGCTTCTGATGGCTC | <i>croquemort (crq)</i> is a receptor involved in phagocytosis and is a marker for plasmacytocytes. |
| <i>He</i> | 5', 3' | CCCGGGCTCGAGGTGCTCGG ATAACGCACACTGCGT GGGGGATCCTGTAACTTAGT GTGAACTGCTCCGCC | <i>Hemese (He)</i> is a transmembrane protein expressed in all cell types. It is thought to have similar actions as the glycoporphins. |
| <i>eater</i> | 5', 3' | CGTCTGTCAATGCCTGACGG AGACACCTTCCAGCTTCGTG | <i>eater</i> is a transmembrane pattern recognition receptor involved in phagocytosis. It is a marker of plasmacytocytes. |
| <i>Hml</i> | 5', 3' | CCGATGATGACGACGAGGAT GATGTTGAAGCTAATGTCCC | <i>Hemolectin (Hml)</i> is a coagulation factor involved in clotting and is a marker of plasmacytocytes. |
| <i>mys</i> | 5', 3' | GTACCATGACCGGAGCAGAT GATCACGGTACATGCGAGTG | <i>myospheroid (mys)</i> is a β -integrin that is a marker of lamellocytes |

| | | | |
|----------------|----|----------------------|---|
| <i>Itgβv</i> | 5' | GGACAGCCTGATCACTGGTT | <i>Integrin βv subunit (Itgβv)</i> is a variant β integrin that is a marker for lamellocytes. |
| | 3' | CTCGCCGGCAACTACTTAAC | |
| <i>cher</i> | 5, | GATCGATGGTCTTCAGGTGC | <i>cheerio (cher)</i> encodes the protein fillamin-240, which is involved in cytoskeleton rearrangement and is a specific marker of lamellocytes. |
| | 3' | CGGATCAGTACGAGGAGAAC | |
| <i>ItgαPS4</i> | 5' | TGAGCACGTTGGTTAGCTTG | <i>Integrin αPS4 subunit (ItgαPS4)</i> is an α integrin, which is a lamellocyte marker. |
| | 3' | ACACCGACTCCTTGACCATC | |
| <i>ItgαPS5</i> | 5' | GCACCCACGTCATAGGAATC | Integrin αPS5 subunit (ItgαPS5) is an α integrin, which is a lamellocyte marker. |
| | 3' | ACTTCGGTTACTCCGTGGTG | |

2.3.7 S2 cell *He (Hemese)* and Oil Red O staining

S2 cells were cultured and treated as described in section 2.3.6 however, cells were cultured in a chamber slide and only treated with Omega fatty acids: Omega-3, -6 and a combination of both. After 48 hours, the cells were fixed with 100 µl 3.7% paraformaldehyde for 10 min. The culture chamber was removed from the glass slide and the slide was washed in 1xPBS for 10 min. Cells were immunostained with mouse MAb H2 anti-Hemese as described in section 2.3.5 with the exception that detergent was omitted from all buffers. Cy5-conjugated anti-mouse IgG (H+L) (Jackson ImmunoResearch laboratories) was used as a secondary antibody at 1:200 dilution and incubated for 60 min, then washes twice in 1xPBS for 10 min. After antibody-staining cells were fixed with 3.7% paraformaldehyde for 10 min, and washed in 1xPBS for 10 min. Oil Red O staining was immediately carried out as described in section 2.3.4. Slides were mounted in Vectashield containing DAPI for confocal microscopy.

2.4 MPO/COX homologue cloning and induction of protein

Irc, Pxn, Pxt as well as human COX-1 and COX-2 (controls) had to be cloned to produce recombinant protein for use in *in-vitro* assays. The amino acid sequences of each gene was obtained from National Center for Biotechnology Information (NCBI)

website. The SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP>) was used to determine the presence and location of a signal peptide and pro-protein cleavage sites for each gene. Serial Cloner 2-5 program was used to search each sequence for restriction enzyme sites to determine which restriction enzymes were best to use.

2.4.1 Bacterial pGEX-2T IPTG induction system

The gene fragments were cloned into the pGEX-2T GST gene fusion vector (GE Healthcare) generating an in frame fusion of protein with GST enabling proteins to be GST tagged for purification. Taking into account the vector and gene sequences, a combination of BamH1 and EcoR1 restriction enzymes was used for cloning (Figure 6). Primers for each gene was designed incorporating a restriction site, and was made to be in frame with protein sequence and vector sequence. *Pxn* is a large gene containing several different domains such as IgGs, so we only cloned the peroxidase catalytic domain located from 557-1165 amino acids. Primers used are shown in Table 5.

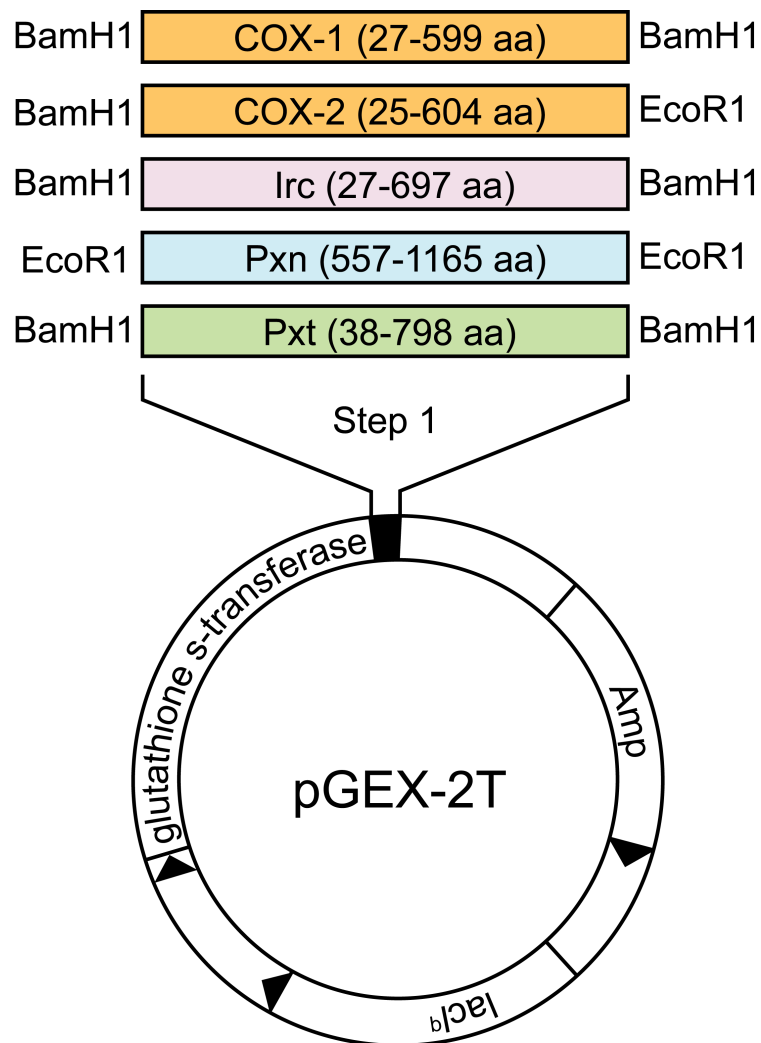


Figure 6. Cloning into the pGEX-2T GST gene fusion vector

Human COX-1 and COX-2 gene sequences, as well as the *Drosophila Irc*, *Pxn* and *Pxt* gene sequences were cloned into the pGEX-2T vector with the restriction enzymes shown. The pGEX-2T vector is a bacterial vector that expresses a fusion protein with GST.

Table 5. Cloning primers for COX-1, COX -2, *Irc*, *Pxn* and *Pxt*

| Gene | Orientation | Restriction enzyme | Primer sequences (5' to 3') |
|--------------|--------------------|---------------------------|--|
| <i>COX-1</i> | 5' | BamH1 | CGC GGATCC GGGGCGCCCACGCCAGTGAAT |
| | 3' | BamH1 | CGC GGATCC TCAGAGCTCTGTGGATGGTCG |
| <i>COX-2</i> | 5' | BamH1 | CCG GGATCC TGTATGAGTGTGGGATTTGACC |
| | 3' | EcoR1 | CGC GAATTC CCTACAGTTCAGTCGAACGTTCTT |
| <i>Irc</i> | 5' | BamH1 | CGC GGATCC AGCCGAGATGTTAATGCCGA |
| | 3' | BamH1 | CGC GGATCC CTAGACGTCTTCACAAGAGAC |
| <i>Pxn</i> | 5' | EcoR1 | CCG GAATTC GTTCCTCGAGGTACAGAAG |
| | 3' | EcoR1 | CCG GAATTC CTTACTTGCCGAGATCTCTTC |
| <i>Pxt</i> | 5' | BamH1 | CGC GGATCC AGCCGAGATGTTAATGCCGA |
| | 3' | BamH1 | CGC GGATCC CTAGACGTCTTCACAAGAGAC |

2.4.1.1 PCR amplification and purification

Gene sequences were amplified using PCR. PCR was carried out using a Veriti 96 well thermal cycler (Applied Biosciences). To amplify, whole adult *Drosophila* cDNA was used for *Irc*, *Pxn* and *Pxt*. HeLa cell cDNA was used for *COX-1* and *COX-2*. 50 µl PCR reactions consisted of: 1x Expand long template PCR, Buffer 1 (Roche), 0.5 mM dNTP, 2 pmol 5' primer, 2 pmol 3' primer, 3.75 units of Expand long template PCR, DNA pol mix (Roche), 1 µg cDNA template. The following program was used: 94°C for 5 min, 35 cycles consisting of: 94°C for 40 sec, X°C (annealing temperature) for 40 sec, 72°C for 3 min, finishing with 72°C for 5 min and continued at 4°C.

Annealing temperatures varied for the different genes; COX-1: 65°C, COX-2: 50°C, Irc: 60°C, Pxn: 55°C and Pxt: 57°C.

PCR fragments were analysed using DNA gel electrophoresis. A 1% agarose gel (150 ml 1xTAE buffer (Tris-Acetate EDTA: 40 mM Tris, 20 mM acetic acid and 1 mM EDTA), 1.5 g Agarose (Fisher Scientific), heated to dissolve, 1x GelRed nucleic stain (BIOTIUM)) was used. Gel electrophoresis was carried out at 120 volts for 25 min. PCR fragments were then purified using a QIAquick PCR gel purification microcentrifuge kit (QIAGEN) according to the manufacturer's protocol. DNA was eluted in 30 µl water.

2.4.1.2 Restriction enzyme digestion, DNA purification and ligation

The purified PCR gene fragments were digested with the corresponding restriction enzymes shown in Figure 6. An 80 µl restriction digestion reaction was used consisting of: 1 µg of DNA (PCR fragment or plasmid), 1xNE4 Buffer (Biolabs), 10 units of either EcoR1, BamH1 (Biolabs) or both for fragments being double digested, and made up to volume with water. Three pGEX-2Tvector digests were also prepared to match the gene fragment digests for cloning. Vectors that were singly digested were treated with 10 units of calf intestinal phosphatase (CIP (Bio Labs)). CIP removes the 5' phosphate group from the DNA strand preventing self-ligation. Reactions were incubated at 37°C for 1.5 hours. Digested DNA fragments were purified using Phenol:Chloroform:Isoamyl alcohol at a ratio of 25:24:1 and pelleted by ethanol precipitation. Pellets were reconstituted in 10 µl water.

The purified digested gene fragments were ligated into their corresponding digested vector using a 10 µl reaction consisting of: a 1:3 molar ratio of vector to insert, 5 units of T4 DNA ligase (Biolabs), 1xT4 ligase buffer made up to volume with water. Ligations were incubated over night at 14°C.

2.4.1.3 DH5 α cell transformation, plasmid extraction and verification

50 μ l DH5 α competent cells (Invitrogen) were added to 5 μ l ligated DNA for each gene ligation, incubated on ice for 30 min and heat-shocked for 1 min at 42°C and immediately placed on ice. 200 μ l lysogeny broth (LB media: 1% Tryptone (Appleton Woods), 1% NaCl (Sigma), and 0.5% Yeast extract (Appleton Woods)) was added and incubated for 1 hour at 37°C. Transformed competent cells were plated onto LB agar plates (LB media with 1.5% Bacto agar) containing 100 μ g/ml ampicillin for selection, and incubated overnight at 37°C. Single colonies from plates were picked and individually cultured in 3 ml LB with 100 μ g/ml ampicillin at 37°C.

To verify transformations, vectors were extracted from cell cultures using a QIAprep Miniprep kit (QIAGEN) following the manufacturer's protocol. DNA was eluted with 30 μ l of water. The vectors were screened by restriction digest (as described before) using a 20 μ l reaction. Digestion products were analysed for vector and gene insert by agarose gel electrophoresis.

2.4.1.4 Sequencing of potential transformed candidates

Genes cloned into the vectors had to be sequenced to confirm that there were no changes in the DNA sequence and genes had been inserted in the correct orientation.

PCR was used for the incorporation of fluorescently labelled Dideoxyribonucleotides (ddNTPs) using Big Dye 3.1 reagent (Applied Biosystems). A 20 μ l reaction for both 5' and 3' primers was used, consisting of 8 μ l Terminator Ready Reaction Mix, 200-500 ng of template, 3.2 pmol of primer (Table 6). The cycle conditions used were: 1 minute at 96°C, 25 cycles consisting of: 10 seconds at 96 °C, 5 seconds at 50 °C and 4 minutes at 60 °C. It finished at 4 °C.

Table 6. GST Primers used to sequence pGEX-2T vectors containing cloned fragments

| Orientation | Primer sequences (5' to 3') |
|--------------------|------------------------------------|
| 5' | GTATATAGCATGGCCTTT |
| 3' | CATGTGTCAGAGGTTTTTC |

Unincorporated dye terminators were removed by washing with 70% ethanol. The pellets were reconstituted in 10 µl of Hi Di Formamide and heated for 95 °C. Sequencing was carried out using capillary electrophoresis. After sequences were checked, positive transformants were bulk cultured to amplify vector and extracted using a Midiprep kit (Qiagen), where the protocol of the manufacturer was followed. DNA was eluted with 150 µl of water and stored at -20°C.

2.4.1.5 Transformation of BL21C cells and IPTG induction

GST expression vectors containing cloned genes had to be transfected into BL21C cells (Stratagene) that have the machinery to produce the proteins.

Transformation of BL21C cells was similar to that used with DH5a cells previously described, except 1 µl of vector containing cloned gene was added to 40 µl BL21C cells. Ampicillin positive colonies from each vector construct were pre-cultured overnight. The pre-cultures were then added to 2 L conical flasks containing: 1 L of LB media, 1 % glucose and 25 µg/ml ampicillin and incubated at 37°C for 3.5 hours. 1 ml aliquots were taken for pre-induction analysis. Isopropyl β-D-1-thiogalactopyranoside (IPTG), the inducing agent, was added to cultures at a final concentration of 0.5 mM. The cultures were incubated further for 2.5 hours at 32°C.

The cells were pelleted by centrifugation at 7900 *g* at 4°C for 5 min. The cells were washed with cold 1xPBS and pelleted. The cell pellets were stored at -80°C.

2.4.1.6 Purification of IPTG induced protein and SDS-PAGE analysis

The pellets were re-suspended in 50 ml of re-suspension buffer (20 mM HEPES buffer (Sigma), 20% glycerol (Sigma), 12.5 M MgCl₂, 2 mM EDTA, 5 mM DTT, 0.1% Igepal CA-630 (sigma) and 1 complete mini protease inhibitor tablet (Roche)). Lysozyme from chicken egg white (sigma) was added at a concentration of 1 mg/ml to lyse cells, and incubated on ice for 30 min. Lysed samples were sonicated on ice for 3 min, at 40% amplitude, at a pulse rate of 6, for four times. The samples were centrifuged at 20,400 *g* for 30 min at 4°C. The supernatant containing the protein was stored on ice. Aliquots of lysed culture after sonication, and supernatant after centrifugation, were collected for analysis.

500 µl Glutathione Sepharose 4B (GE Healthcare) was added to a 10 ml column and equilibrated with 10 ml re-suspension buffer. The supernatant containing the induced protein was added to the column and allowed to filter through. The column was washed with 50 ml wash buffer (Re-suspension buffer containing 0.15 M KCL) to elute weakly bound proteins from the resin. To elute bound GST-tagged proteins, 10 ml of elution buffer (Re-suspension buffer containing 0.15 M KCL and 25 mM L-Glutathione reduced (Sigma)) was added to the column and 5x 1 ml elution samples were collected which should contain GST-tagged proteins. Protein induction and purification analysis was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A 1:1 ratio of sample and loading buffer (NOVEX Tris-glycine SDS sample buffer 2x (Invitrogen) containing 2-Mercaptoethanol (Sigma)) was made and incubated at 95°C for 5 min. 40 µl of each sample and 5 µl of Precision plus protein (BIO-RAD) protein

ladder was added to an 8% SDS gel and ran for 90 min at 125 volts in 1x SDS-PAGE running buffer (25 mM Tris (Sigma), 250 mM Glycine (sigma) and 0.1% SDS, pH 8.3). The SDS gel was then stained with Brilliant Blue R Concentrate (Sigma) for 5 min and de-stained overnight destain buffer (10% acetic acid glacial (BDH) and 40% methanol). The gels were dried in cellophane sheets (Invitrogen) for preservation.

2.4.2 HeLa cell pTRE3G doxycycline inducible system

Although GST fusion proteins were induced in bacteria, all protein was insoluble. To obtain soluble protein we switched to expression in human HeLa cells. As a first step, we modified the pTRE3G vector to include 3xFLAG (GAC TAC AAA GAC CAT GAC GGT - GAT TAT AAA GAT CAT GAC ATC - GAT TAC AAG GAT GAC GAT GAC AAG) optimised for detection with monoclonal Anti-FLAG antibody (Sigma) as well as a BamH1 and EcoR1 restriction site. Gene fragments from the pGEX-2T vectors were sub-cloned into pTRE3G-FLAG vector containing 3xFLAG insert (Figure 7).

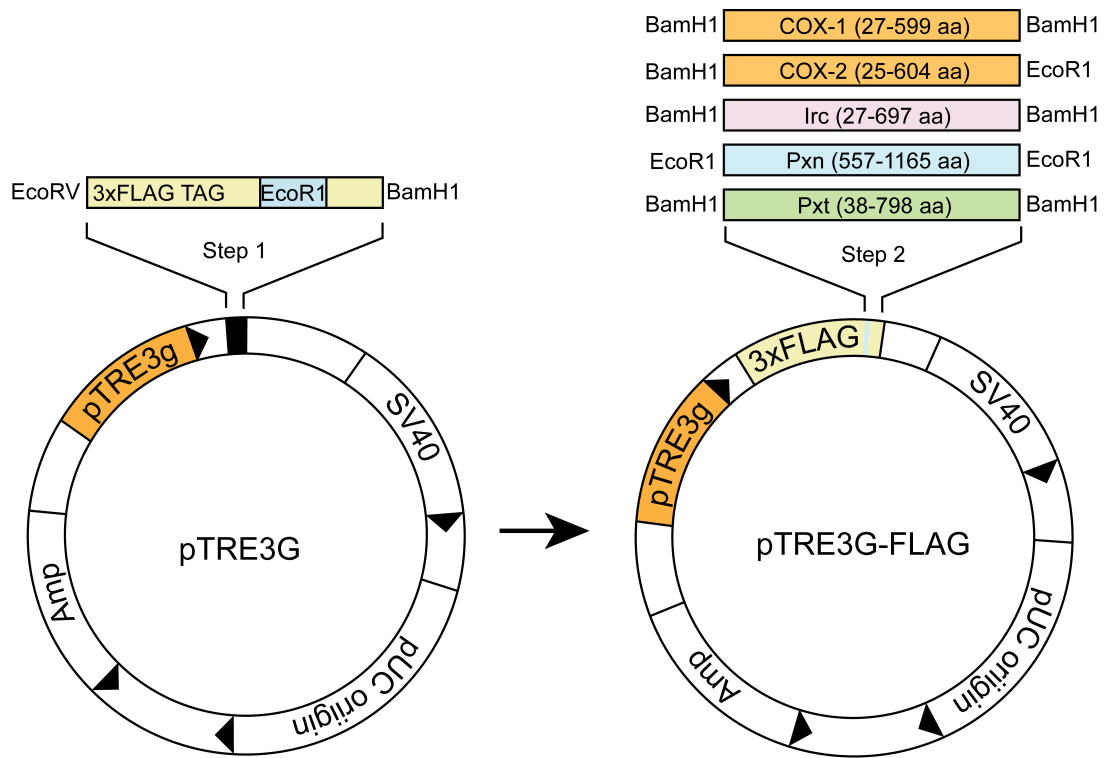


Figure 7. Cloning into a pTRE3G expression vector

A 3xFLAG insert was cloned into the pTRE3G vector using EcoRV and BamH1 restriction enzymes. Gene sequences were restriction digested from pGEX-2T vector (as previously described) and the gene fragments sub-cloned into pTRE3G-FLAG vector using a combination of BamH1 and EcoR1 restriction enzymes.

The FLAG tag insert was made by annealing two primer sequences (as shown in Table 7). A 50 µl reaction mix was used to anneal the two sequences: 1xNE4 ligation buffer, 2 pmol of 5' primer, 2 pmol of 3' primer and made up to volume with water. This was placed in the following PCR program: 95°C for 5 min, 95°C for 0.15 sec, 37°C for 1 min and finishing at 10°C for 0.30 sec.

Table 7. Primers used to create 3xFLAG-tag insert

| Orientation | Primer sequences (5' to 3') |
|--------------------|---|
| 5' | ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACAT CGATTACAAGGATGACGATGACAAGAATTCCCGGG |
| 3' | GATCCCCGGGAATTCTTGTCATCGTCATCCTTGTAATCGATGTC ATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCAT |

2.4.2.1 Insertion of 3xFLAG insert into pTRE3G vector

pTRE3G vector was restriction digested with EcoRV and BamH1 in a 80 µl reaction consisting of: 80 µl restrictive digestion reaction was used consisting of: 1 µg of DNA (3xFLAG insert or vector), 1xNE4 Buffer (Biolabs), 10 units of EcoRV and 10 units of BamH1 (Biolabs). The restriction digest was incubated at 37°C for 1.5 hours and then purified using Phe/ChI/IAA, precipitated with ethanol and the pellet was reconstituted in 10 µl water.

Digested pTRE3G vector and 3xFLAG-tag insert were ligated in a 10 µl ligation reaction consisting of: 1:5 molar ratio of vector:3xFLAG insert, 10 units of T4 DNA ligase (Biolabs), 1xT4 ligase buffer made up to volume with water. Ligations were left over night at 14°C. The ligated products were transfected into DH5α competent cells and was plated on to an LB agarose gel containing ampicillin. Eight ampicillin

resistant colonies were picked and cultured separately. The vectors were extracted from each culture using a QIAprep Miniprep kit. DNA from each culture was digested with Ecor1 because only the vectors containing the FLAG-tag insert will digest. A 20 µl restriction digestion reaction was carried out and the digestion products were analysed on a 1% agarose gel. Vectors that were positive for 3xFLAG insert were sequenced. The primers used for sequencing are shown in Table 8. A Midiprep of the correct clone was carried out and stored at -20°C.

Table 8. pTRE3G primers used to sequence pTRE3G-FLAG vector

| Orientation | Primer sequences (5' to 3') |
|--------------------|------------------------------------|
| 5' | GAAAGGCTACCACTTCCTACCCTCGTAAAGTC |
| 3' | CTGAGAGTTGTAAGATGAGGAGGT |

2.4.2.2 Insertion of MPO/COX genes into pTRE3G

The pTRE3G-FLAG vector was digested in three ways A) EcoR1 alone, B) BamH1 alone, and C) EcoR1 and BamH1. Single restriction enzyme cut vectors, were treated with CIP to prevent self-ligation. The MPO/COX cloned genes were digested out of the pGEX-2Tvector to be sub-cloned into the pTRE3G-FLAG vector using their corresponding restriction enzymes. Restriction digestions were carried out using an 80 µl reaction as described previously. Digestions were purified using Phe/ChI/IAA and reconstituted in 10 µl water. Gene fragments were then ligated into digested vectors with corresponding restriction enzyme digests in a 10 µl reaction as previously described.

Ligated products were used to transform DH5 α competent cells and were plated on to LB agarose plates containing ampicillin for selection. Eight colonies were picked for each vector construct and cultured separately. The vectors were extracted from each culture using a QIAprep Miniprep kit. Miniprep DNA from cultures was digested using a 20 μ l reaction with the corresponding restriction enzymes for each gene fragment. Digestions were analysed on a 1% agarose gel. The vectors containing cloned genes were sequenced. Primers used for sequencing are shown in Table 8. Vectors containing the correct insertions were amplified by plasmid Midiprep and stored at -20°C.

2.4.2.3 HeLa cell culturing

HeLa cells were incubated at 37°C with 5% CO₂ and cultured in Dulbecco's Modified Eagle's Medium (DMEM). 500 ml of DMEM was modified to contain 10% FBS, 1x Penicillin-Streptomycin (Sigma), 1x GlutaMAX (GIBCO) and 1x non-essential amino acids (Sigma).

HeLa cultures were grown for 3 days before being split into a new culture flask. To split cells, media was removed and cells washed with 5 ml 1xPBS. Cells were detached by incubating for 5 min with 1x Trypsin-EDTA (Sigma). The flask was tapped to dislodge cells from the surface. DMEM was added to detached cells to neutralise trypsin. 2 ml of detached cells were transferred to 18 ml of fresh DMEM media in a T75 flask (Corning).

2.4.2.4 Lipofectamine transfection of pTRE3G-FLAG expression vectors into HeLa cells

A day before the transfection, 6x 500 μ l HeLa cells were plated out into a 24 well plate (Corning) so at the time of transfection, they will be 50-80% confluent. 1.5 μ g pCMV-Tet3G activator vector and 250 ng of pTRE3G-FLAG expression vector for

each gene was added to 100 μ l serum-free media. 8 μ l of Lipofectamine LTX (Invitrogen) was added to each transfection mixture and incubated at room temperature for 30 min. The DNA-lipid mixture was added drop by drop to the wells containing cells and mixed by rocking plate back and forth. Transfected cells were incubated at 37°C with 5% CO₂ overnight. The serum free media was removed and replaced with serum-containing media with 300 μ g/ml Neomycin (Sigma) for selection. Cells were bulked and transferred to a T75 flask where they were maintained. Transformed cells were selected against by using 300 μ g/ml and eventually bulked to make stable lines.

2.4.2.5 Doxycycline induced protein and analysis by Western Blot

The five HeLa transfected stable lines were freshly split and grown to a confluence of 80%. A final concentration of 1 μ g/ μ l of Doxycycline was added to induce protein production for 24 hours. Induced cells were harvested using Trypsin. Detached cells were pelleted by centrifugation at 4900 g for 10 min at 4°C. The media was removed and the cells were washed with 1xPBS. The pelleted cells were re-suspended in 80 μ l of 1xRIPA buffer (Sigma) containing protease inhibitors and incubated for 30 minutes on ice to lyse the cells. Lysed cells were centrifuged at 4900 g for 10 min at 4°C. The supernatant was transferred to a new tube and kept on ice.

The protein concentration of the supernatant for each induced protein was measured using 1x Bradford Assay (BIO-RAD) and the absorbance measured using a spectrophotometer. The line equation was used from a standard curve previously made with known protein concentrations, to calculate the concentration of each induced protein extract was calculated.

50 μ g of extract from each induced protein line was ran on an 8% SDS-PAGE gel as described in section 2.4.1.6, for HeLa (control), COX-1, COX-2, Pxn, Pxt and Irc. An

X Cell II Blot Module (Invitrogen) was used to transfer protein from the SDS-PAGE gel to a western membrane. The module was prepared by placing two sponges and a piece of filter paper soaked in 1x transfer buffer (25 mM Tris, 192 mM Glycine, and 20% methanol) on top of one another. The SDS-PAGE gel was placed on top and a blotting membrane (Whatman), activated in methanol and equilibrated in 1x transfer buffer was placed on top. A further filter paper and two sponges soaked in 1x transfer buffer was placed on top of this and the other half of the module secured. The western transfer was run at 30 volts for 1.5 hours in 1x transfer buffer.

After transferring, the membrane was blocked overnight in 1xTBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.1% Tween 20) containing 5% non-fat dry milk. Antibody staining was also performed in the same buffer containing 1:1000 Monoclonal mouse Anti-FLAG M2 antibody (Sigma) and was incubated at room temperature for 1.5 hours. The membrane was washed three times for 10 min with 1xTBST and was incubated with the secondary antibody Anti-mouse HRP IgG (H+L) (PROMEGA) using a 1:10,000 dilution and incubated for 1 hour at room temperature. The membrane was then washed three times with 1xTBST for 10 min. The membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min. A series of exposures were then processed. 1:10,000 Monoclonal mouse Anti-GAPDH (PROMEGA) antibody was used as a control.

After identifying correct protein induction conditions, a large-scale induction was done. The five transgenic HeLa lines and HeLa (control) cells were cultured in a T150 flask (Corning) and expression induced with 5 µg/µl of Doxycycline over 48 hours. After 24 hours, media was discarded and fresh media with Doxycycline was added. The cells were trypsinised, and pelleted by centrifugation at 4900 g for 10 min at 4°C. Cells were washed with 1xPBS and the cell pellets were then stored at -80°C.

2.4.2.6 Purification of FLAG-tagged induced proteins and elution with FLAG peptide

180 μ l (30 μ l x6) of Dynabeads Protein G (Life Technologies) was washed five times with 1xPBS 0.1% Tween-20 (PBST). Beads were re-suspended with 500 μ l PBST containing 30 μ l (30 μ g) Monoclonal Anti-FLAG M2 antibody. The beads were rotated overnight at 4°C, and then washed five times with PBST and reconstituted in 120 μ l of PBST and stored on ice.

1ml 1xRIPA buffer was added to the cell pellets for COX-1, COX-2, Pxn, Pxt and Irc and HeLa for control and incubated on ice for 30 min. The lysates were sonicated six times for 30 sec at 40% amplitude and at a pulse rate of 6. The samples were centrifuged at 4900 g for 15 min at 4°C. 20 μ l of the Ab covered beads were added to each lysate and incubated for 3 hours at 4°C. The beads were washed three times with 1xRIPA and then with 100 mM Tris-HCL, 100 mM NaCl of pH 8.0 containing 20% glycerol.

FLAG-tagged protein was eluted from the beads by adding 20 μ l 100 μ g/ml 3x FLAG Peptide (Sigma) (dissolved in 100 mM Tris-HCL, 100 mM NaCl of pH 8.0 containing 20% glycerol), and left to incubate on ice. Beads were pelleted and the supernatant removed to a fresh tube. Elution was repeated, and the supernatants were pooled to give a total volume of 40 μ l. Eluted FLAG-tagged protein was stored at -80°C.

2.4.2.7 *In vitro* COX activity assay

A COX fluorescent activity assay kit (Cayman) was used to determine cyclooxygenase activity. The assay indirectly measures cyclooxygenase activity by relying on the formation of PGG₂, and then measures the peroxidation step of this to its alcohol form PGH₂.

A 1:5 dilution of each FLAG-tagged purified protein was used in the assay, where COX-1 and COX-2 FLAG-tagged proteins were used as positive controls in comparison with the COX standard internal control. The assay was carried out according to the manufacturer's protocol using a Fluoroskan (Ascent 1.6 Labsystems) with an excitation wavelength of 550 nm and an emission wavelength of 615 nm. Ascent research addition 1.2.3.1 program was used to analyse the data.

We modified the assay to also measure at peroxidase activity. Arachidonic acid was substituted in the assay for 10 µl 5 mM hydrogen peroxide, making a final concentration of 0.27 mM.

2.4.3 *In vivo* characterisation of MPO/COX homologues

Natural longevity and survival after infection with pathogens were carried out using the following knock-out fly strains: *Pxt*^{f05258}, *Irc*^{MB11278} and for *Pxn*, *Pxn* targeted RNAi (Transformant ID: 15276 VDRC) knock down in blood cells driven by *P{UAS-Dcr-2.D}1*, *w*¹¹¹⁸; *P{Hml-GAL4.Δ}2*, *P{UAS-2xEGFP}AH2*. *w*¹¹¹⁸ and the driver alone were used as controls.

For each fly strain, five replicates of 50 male flies, in total 250 flies were used per assay. Male *Drosophila* from each strain was aged between 2-5 days after eclosion before use in assays. Data from each assay were input into Excel to be analysed. Kaplan-meier graphs were produced using the program SPSS.

2.4.3.1 Natural longevity Assay

Drosophila were incubated at 25°C and their survival checked and recorded each day. The vial they were contained in was also changed daily.

2.4.3.2 Natural infection with *Beauveria bassiana*

B.bassiana (CABI) is a pathogenic fungal strain. It was cultured on carrot and potato agar media and allowed to grow for 3 days when it reached peak sporulation. Flies from each genetic strain were anaesthetised using CO₂. For the control group, for each rep, flies were placed onto a sterile potato and carrot agar plate and shaken gently over the plate for 10 seconds and then placed into a fresh vial. For the infected group, flies were treated in the same way, but shaken over the *B.Bassiana* grown potato and carrot agar plate.

Potato and carrot agar plates are made from one small potato and carrot grated and boiled in 1 L of water. It is then strained and made up to 1 L with water. This potato and carrot containing solution is substituted in place of water in the normal LB agar recipe.

2.4.3.3 Wounded infection with *Enterococcus faecalis*

E.faecalis was a gift from Bruno Lemaitre and is a Gram +ve bacterium. *E.faecalis* was plated out onto an LB agar plate and incubated overnight at 37°C. A colony was picked and cultured in 3 ml of LB overnight at 37°C on in shaking incubator. The culture was diluted to make a working stock of OD₁₀. The Optical density of the culture was measured using a spectrophotometer. Flies were anesthetised on a CO₂ pad and wounded between the A5 and A4 dorsal abdominal tergites with a fine sterilised platinum needle with either 1xPBS for control or bacteria culture. After wounding, flies were placed into a fresh vial and the survival recorded every 24 hours.

2.4.3.4 Wounded infection with *Pseudomonas entomophila*

P.entomophila was a gift from Huw Williams and it is a Gram –ve bacterium. *P.entomophila* was cultured on LB agar plates containing 10% non-fat dried milk

30°C. A colony with a clear halo was pre-cultured in 3 ml LB at 30°C in a shaking incubator. 1 ml of the pre-culture was added to a final volume of 200 ml LB in a 1 L conical flask and incubated over night using the same conditions as the pre-culture. The OD of the culture was adjusted to make a working stock of OD_{0.1}. Infection of flies was carried out as described with *E.faecalis*. The survival of infected flies was assayed every 6 hours.

2.4.3.5 RT-PCR analysis of infected *Drosophila* strains

RT-PCR analysis was used to determine changes in expression of a panel of known immune-induced genes shown in Table 9.

5 male flies were collected for both control and infected flies for each survival assay. For *B.bassiana*, 5 flies were collected every 24 hours for 0-5 days. For *E.faecalis*, 5 flies were collected every 24 hours for 0-4 days. For *P.entomophila*, 5 flies were collected every 6 hours for 0-12 hours. Three repetitions per time point were taken. Flies were stored at -80°C. The RNA isolation, conversion to cDNA, and RT-PCR reactions were carried out as described previously. *RpL32* was used as the endogenous control to normal expression to for all analyses.

Table 9. Primers used to analyse Infection assays

| Gene | Orientation | Primer sequences (5' to 3') |
|--------------|--------------------|------------------------------------|
| <i>RpL32</i> | 5' | CAACAGAGTCGGTCGCCGCTTCAACGGACA |
| | 3' | CAGCTCGCGCACGTTGTGCACCAGGAACTT |
| <i>Pxt</i> | 5' | GCAGCTCCTCGATGTGATTGAAAC |
| | 3' | CTAGAGTGCGAGCGAGAGGTAAGA |
| <i>Irc</i> | 5' | TGATCTACACGATCTTCATGC |
| | 3' | CTCCTCAATGACCACTCGACG |
| <i>Pxn</i> | 5' | CACCCGAACAACCTGACCCAAA |
| | 3' | CGATTATATCCTCGCACTTCT |

3. Results

Inflammatory disease is on the rise due to a combination of lifestyle, environment and genetic predisposition. Inflammatory diseases have extensive affects on health and economic costs. There is a requirement for new therapeutic targets to be developed and *in vivo* models that can be used to dissect underlying inflammatory mechanisms. Mouse models are currently the go-to for *in vivo* modelling of inflammatory diseases such as atherosclerosis and rheumatoid arthritis (Daugherty, 2002; Asquith et al., 2009). We believe *Drosophila* could be a useful genetically tractable, cheap alternative tool in aiding inflammatory research.

We have used the *hop*^{Tum} mutant to initiate an inflammatory response, by overstimulating the JAK/STAT pathway resulting in the formation of an inflammatory melanotic tumour (an aggregation of blood cells) (Harrison et al., 1995). *hop*^{Tum} *Drosophila* have allowed us to dissect downstream lipid mediators that potentiate inflammation. We have shown that *Drosophila* provides an ideal model to characterise function and molecular mechanisms of lipid mediators.

3.1 Screening for enhancers and suppressors of inflammatory tumours

The use of the *hop*^{Tum} mutant as a model to instigate an inflammatory insult enabled us to assay for conditions that can either enhance or reduce the inflammatory tumour phenotype. The *hop*^{Tum} mutant causes constitutive signalling of the JAK/STAT pathway that results in over-proliferation of blood cells, and lamellocyte differentiation. This culminates in the production of inflammatory tumours. We speculated that lipid mediators, such as prostaglandins, play a role in the production of these tumours, and that the *hop*^{Tum} mutant could be used to elucidate factors that influence the production of downstream lipid mediators.

3.1.1 The effect of dietary supplementation of lipids on tumour incidence

By supplementing *Drosophila* media with omega-3 and omega-6, we wanted to see if we could enhance or suppress the incidence of the *hop*^{Tum} melanotic tumour phenotype. Omega-3 provides a source of α -linolenic acid, and omega-6 provides a source of linoleic acid, precursors to lipid mediators. These results would demonstrate whether production of downstream lipid mediators could be increased or reduced.

Safflower oil contains 64% of linoleic acid, an unsaturated omega-6 fatty acid that is a precursor of arachidonic acid (Figure 2). It was anticipated that supplementation of linoleic acid would result in an increase in pro-inflammatory mediators by increasing the availability of their precursor. We used four different concentrations of linoleic acid and incubated the flies at a lower temperature of 25°C. Because the *hop*^{Tum} mutant is temperature-sensitive-conditional, a lower temperature will reduce induction of melanotic tumours, allowing for it to be used to assay for enhancers of the phenotype. We observed that supplementation of omega-6 significantly

enhanced tumour incidence. A concentration of 0.3% resulted in an increase of tumour incidence of 13.5% compared to control (Figure 8A). At a concentration of 0.7% and higher, the incidence plateaued, but was still markedly increased compared to the control. At higher concentrations, effects were obstructed by lethality.

In contrast, α -linolenic acid, an unsaturated omega-3 fatty acid that is a precursor of EPA (Figure 2) and is known for its anti-inflammatory properties. Supplementation with α -linolenic acid is therefore expected to reduce tumour incidence due to the production of anti-inflammatory lipid mediators. We used four different concentrations and incubated flies at 27°C to be able to detect suppression of tumour incidence. As expected, α -linolenic acid supplementation between 0-0.7% reduced overall tumour incidence by 43.8% (Figure 8B). We observed that α -linolenic acid supplementation was able to counteract the effects of linoleic acid supplementation; by supplementing media with a constant concentration of 0.15% of linoleic acid, and adding increasing amounts of α -linolenic acid we were able to reduce tumour incidence by 33.4% (Figure 8C).

To determine whether the effects seen with linoleic acid and α -linolenic acid were unique to polyunsaturated fatty acids, we assayed both saturated fatty acids and cholesterol. These two types of lipid have been connected to inflammatory diseases like cardiovascular disease. Coconut oil contains 85% saturated fatty acids and was anticipated to enhance the inflammatory tumour phenotype. Saturated fatty acid was supplemented at five different concentrations and the flies incubated at 25°C to assay for enhancement of inflammation. Saturated fatty acid failed to significantly increase tumour incidence across all concentrations compared to the control (Figure 8D). Cholesterol however, when assayed in the same way reduced tumour incidence

by 8% at a concentration of 0.005% but increased again from 0.01-0.05%, suggesting its effects may be dose dependent (Figure 8E).

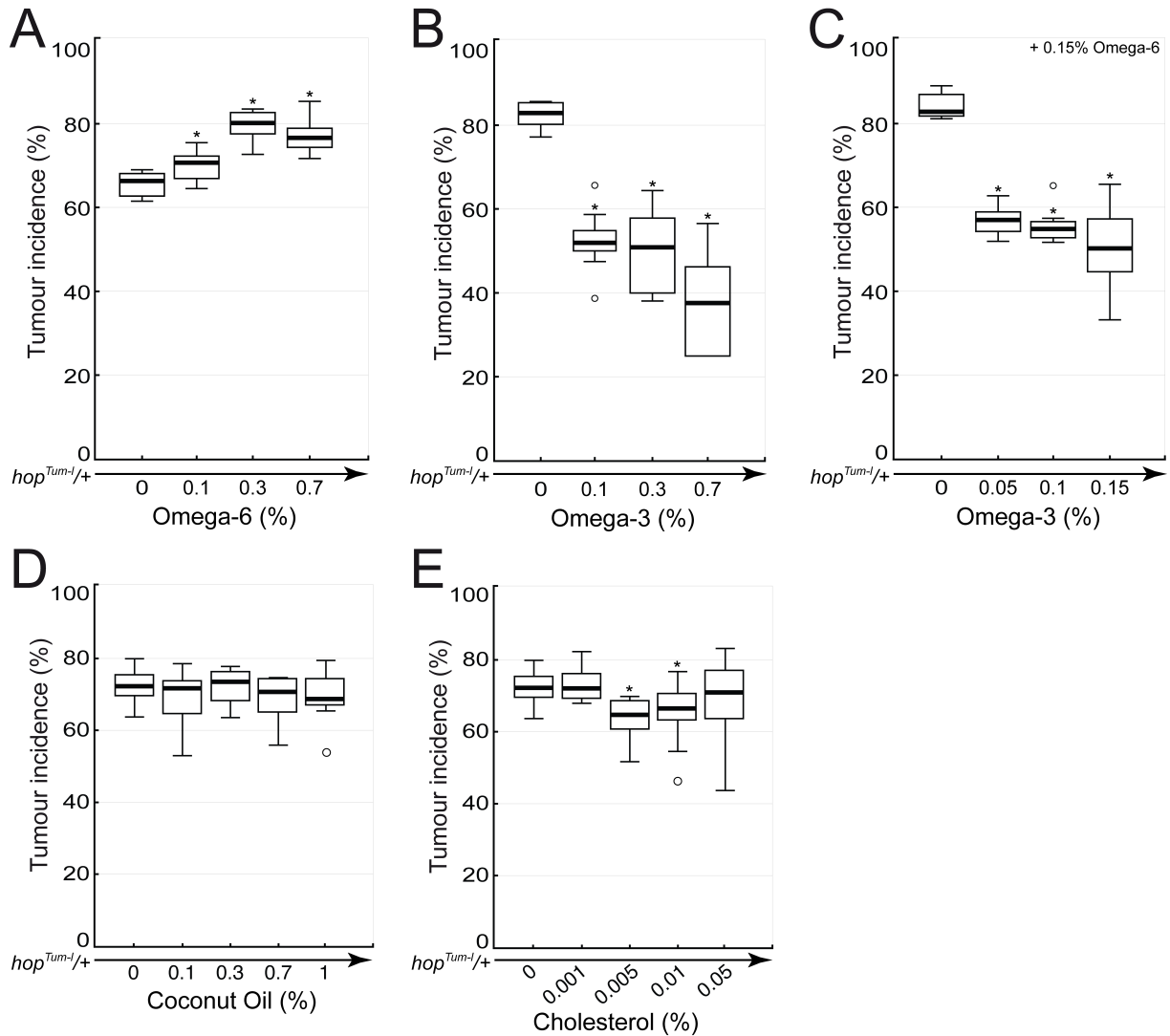


Figure 8. Effects of dietary supplementation of lipids on inflammatory tumour incidence

Drosophila yeast-agar media was supplemented with different lipids to assay the effects on tumour incidence in *hop*^{Tum} heterozygous females. 0% represents non-supplemented media (A) Supplementation with the omega-6 fatty acid, linoleic acid (%). (B) Supplementation with the omega-3 fatty acid, α -linolenic acid (%). (C) Supplementation with a constant concentration 0.15% of linoleic acid with increasing α -linolenic acid supplementation. (D) Supplementation with saturated fatty acid. (E) Supplementation with cholesterol. A, D and E were incubated at 27°C to assay for enhancement in tumour incidence. B and C were incubated at 25°C to assay for suppression of tumour incidence. * Indicates those values that are statistically significant having a P-value less than 0.001.

3.1.2 Effects of fatty acid liberating pathway knockouts on tumour incidence

Our results have identified that the *hop^{Tum}* phenotype can be influenced by supplementation of key precursors of lipid mediators, such as prostaglandins. These key precursors are esterified to form triglycerides that are stored in the phospholipid membrane, which are then cleaved to produce free fatty acid. We next sought to elucidate the pathway components that are involved in the liberation of free fatty acid, making them available to be processed into inflammatory lipid mediators.

We selected key components in the well-established arachidonic acid (free fatty acid) synthesis pathway in mammals that includes the PLA2 and the PLC pathway, and searched the *Drosophila* genome for homologues using BLAST (Altschul et al., 1990). We observed that the *Drosophila* genome lacks homologues for mammalian cytosolic phospholipase A2 (cPLA2). However, *secretory PLA2 (sPLA2)* was detected as were three homologues of phospholipase C, Phospholipase C at 21C (Plc21C), No receptor potential A (NorpA) and Small wing (SI). As shown in Figure 3, PLA2 can directly hydrolyse free fatty acid from the phospholipid membrane. PLC hydrolyses PIP2 to liberate diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP₃). DAG may then be hydrolysed by DAG lipase into monoacylglycerol and AA (Figure 3). Knockout flies in *sPLA2*, *small wing*, *norpA*, and *PLC21C*, as well as *Drosophila* homologues of the downstream PLC components (described in Table 3) were selected and tested for potential effects on inflammatory tumour incidence.

We tested whether reducing sPLA2 and PLC levels would reduce fatty acid production and therefore inflammatory tumour number. We expected the knockouts to reduce tumour incidence, so flies were incubated at 27°C to assay for suppression. The strongest sPLA2 mutant, *Df(2R)BSC263* gave an impressive 42.1% reduction in tumour incidence compared to the control (Figure 9A). Similarly,

the Small wing mutant *sl²* also reduced tumour incidence by 38.71% (Figure 9A). However, the greatest suppression was seen with mutations in the other two PLC homologues, *Plc21C*, which decreased tumour incidence by 72.8%, and *norpA*, which reduced tumour incidence by 71.8% (Figure 9A).

Due to the significant suppression of tumours seen with the PLC mutants, we tested further deletions of PLC signalling pathway components. We expected PLC pathway knockouts to also reduce tumour incidence, as they would prevent the availability of free fatty acid. Phosphatidylinositol phosphate kinase (PIPK5) is known to phosphorylate phosphatidylinositol 4-phosphate (PI4P) to produce PIP2, which regulates its availability in the phospholipid membrane. Using mutations in the *Drosophila* PIPK5 homologue Skittles, a reduction in tumour incidence of 20.9% was achieved (Figure 9A). Similarly, mutations in Gαq, a G protein that activates PLC, reduced tumours by 36.6% (Figure 9A). Finally, by reducing expression of the *Drosophila* DAG lipase homologue InaE to reduce the hydrolysis of DAG to monoacylglycerol (MAG) and AA reduced tumour number by 20.4% (Figure 9A). In contrast, mutations in the *Drosophila* homologue diacylglycerol kinase (DAGK) RdgA, increased tumour incidence by 47.8% (Figure 9A). DAGK is the enzyme that catalyses the conversion of DAG to phosphatidic acid (PA) and can control the availability of DAG. By reducing DAGK levels, we speculate that DAG levels are stabilised, resulting in the increased availability of DAG, leading to increased AA production.

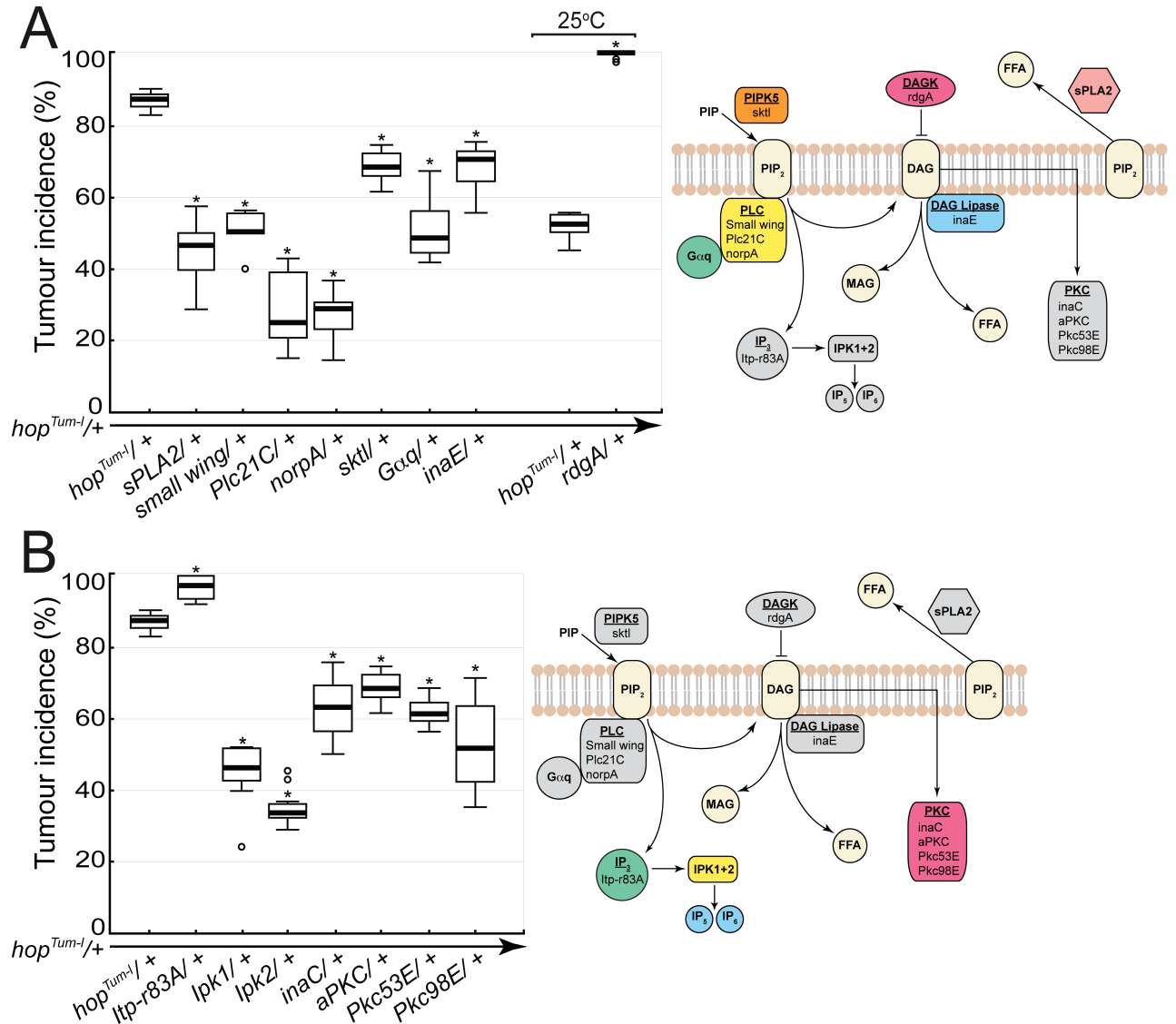


Figure 9. Effects of fatty acid liberating pathway knockouts on tumour incidence

Heterozygote females for mutant/*hop^{Tum}* for each pathway component were assayed for effects on tumour incidence (%). Flies were incubated at 27°C to assay for suppression of tumours, except for in the case of *rdgA* where 25°C was used to assay for enhancement of tumours. (A) The following knockouts of PLA2 and PLC pathway components were used: *sPLA2* (*Df(2R)BSC263*), *small wing* (PLC; *sl²*), *norpA* (PLC; *norpA⁴⁵*), *Plc21C* (PLC; *Plc21C^{KG08451}*), *skt1* (PIPK5; *skt1^{Δ15}*), *Gαq* (*Ga49B¹*), *inaE* (DAG lipase; *inaE^{X118}*) and *rdgA* (DAGK; *rdgA¹*). (B) The following knockouts of signalling components downstream of PLC were used: *ltp-r83A* (IP₃ receptor; *ltp-r83A^{SV35}*), *lpk1* (IPK1; *P{EPgy2}^{EY04862}*), *lpk2* (IPK2; *P{GSV6}^{GS15129}*), *inaC* (PKC; *Df(2R)BSC382*), *aPKC* (PKC; *P{lacW}aPKC^{k06403}*), *Pkc53E* (PKC; *P{EPgy2}Pkc53E^{EY14093}*), *Pkc98E* (PKC; *Df(3R)Exel6211*). Several mutant strains were used for each gene (Table 3), only data from the strongest allele is shown. * Indicates those values that are statistically significant having a P-value less than 0.001.

PLC is not only involved in the liberation of free fatty acid, it plays a role in a number of other downstream signalling pathways such as calcium signalling through inositol 1, 4, 5-trisphosphate (IP_3). We clarified whether the effects seen with PLC knockout was due to failure to liberate free fatty acid or effects on calcium signalling pathways. PLC hydrolyses PIP₂ to produce DAG and IP_3 , IP_3 can bind to the receptor Inositol 1,4,5-trisphosphate receptor (Itp-r83A) resulting in intracellular calcium release. Reduction in Itp-r83A did not suppress the tumours, but actually induced the number by 7% (Figure 9B). IP_3 can also be further phosphorylated by the kinases IPK1 and IPK2 to generate a number of inositol phosphate variants, which have been implicated in signalling (Berridge and Irvine, 1989). Reduction in levels of IPK1 that produce inositol pentakisphosphate (IP_5), and IPK2, that produces IP_6 , reduced tumour incidence by 41.4% and 50.7% (Figure 9B).

Another route, through which the PLC pathway can act, is via activation of protein kinase C (PKC) by DAG. To determine if PKC signalling affects tumour incidence we used mutants for the *Drosophila* PKCs. Mutations in Inactivation no afterpotential C (inaC) and Protein C kinase 53E (Pkc53E) reduced tumour incidence by 22.1% and 23.41% respectively (Figure 9B). Whereas, mutations in atypical protein kinase C (aPKC) and Protein C kinase 98E (Pkc98E) suppressed tumours by 30.1% and 34% (Figure 9B).

3.1.3 Effects of MPO and COX inhibitors on inflammatory tumour incidence

Supplementation of fatty acids suggested that a common precursor was being utilised to produce pro-inflammatory lipid mediators. We therefore wanted to determine if we could reduce the inflammatory phenotype by using inhibitors of both cyclooxygenase (COX) and myeloperoxidase (MPO). These are two enzymes known to produce pro-inflammatory lipid mediators. Inhibition of these reduces inflammation (Liu et al., 2012; Vane, 1971). The effects of non-specific COX inhibitor (Aspirin and

Indomethacin), COX-1 specific (SC-560), COX-2 specific (NS-398) and MPO (MPO-1) inhibitor on the tumour incidence were investigated. Concentrations above 3 mM had no greater effect. A net tumour suppression of 42.1% was shown between 0-10 mM (Figure 10A). The same effect was seen when treated with the non-specific COX inhibitor indomethacin (data not shown). Suppression by aspirin and indomethacin raised the possibility of COX activity being present. To distinguish whether this COX activity corresponds to a COX-1 or COX-2 activity, we used specific isoform COX inhibitors. When using a COX-1 specific inhibitor SC-560, tumour incidence was reduced by 33.2% (Figure 10B). The COX-2 specific inhibitor NS-398 reduced tumour incidence by 35.9% (Figure 10C). Because COX is a member of the larger MPO family, we used an MPO inhibitor that inhibits the peroxidase activity of MPO (COX also has peroxidase activity). Treatment with MPO Inhibitor-1 also showed a marked reduction in tumour incidence between concentrations 0-50 nM of 29.7% (Figure 10D).

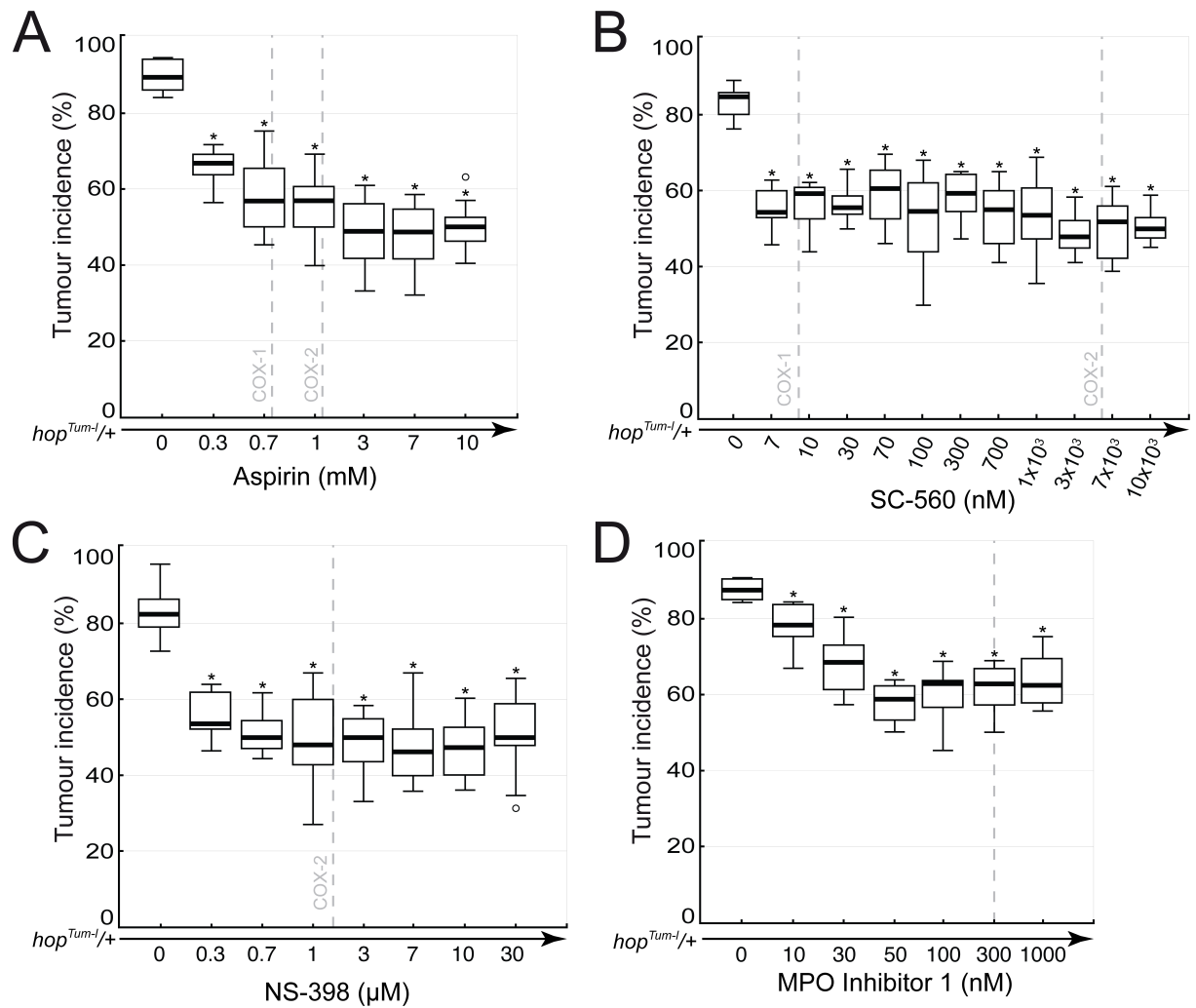


Figure 10. Effect on tumour incidence after treatment with MPO and COX inhibitors

Inhibitors were supplemented at concentrations that span the known IC₅₀ of the drug used. Concentration 0 corresponded to vehicle alone (DMSO) and was used to compare the effects seen with inhibitors. The effects of tumour incidence were assayed in heterozygous *hop*^{Tum} flies. (A) Aspirin, a non-selective COX inhibitor. (B) SC-560, a COX-1 specific inhibitor. (C) NS-398, a COX-2 specific inhibitor. (D) MPO Inhibitor-1, an inhibitor of MPO peroxidase activity. Dashed lines show the published IC₅₀ of the inhibitor for its specific enzyme. * Indicates those values that are statistically significant having a P-value less than 0.001

For each inhibitor, we calculated the experimental IC₅₀ i.e. the concentration needed to reduce tumour incidence by a half. A logarithmic curve was fitted to the data points and the IC₅₀ subsequently calculated from the resultant line equation and compared to the published IC₅₀ values for each drug (Table 10). The calculated IC₅₀ can elucidate function of the enzyme activity present, showing it to be COX-1, COX-2 or MPO. All inhibitors established a lower IC₅₀ than the published values.

Table 10. Calculated IC₅₀ in comparison to published IC₅₀ values

| Inhibitor | Published COX-1 IC ₅₀ | Published COX-2 IC ₅₀ | Published MPO IC ₅₀ | Experimental IC ₅₀ |
|-----------------|----------------------------------|----------------------------------|--------------------------------|-------------------------------|
| Aspirin | 0.75 mM | 1.25 mM | - | 0.18 mM |
| SC-560 | 9 nM | 6.3 µM | - | 1.05 nM |
| NS-398 | 75 µM | 1.77 µM | - | 0.08 µM |
| MPO Inhibitor-1 | - | - | 300 nM | 19.71 nM |

3.1.4 Effect of *Drosophila* MPO/COX homologue knockouts on tumour incidence

The reduction in the incidence of inflammatory tumours by both MPO and COX inhibitors indicated that COX or MPO activity is present in *Drosophila*. Inhibition by COX and MPO reduced the production of pro-inflammatory lipid mediators resulting in suppression of tumours. We sought to identify *Drosophila* MPO and COX homologues that could account for this potential activity by BLAST. The BLAST search identified eight potential MPO/COX homologues for COX-1, COX-2 and MPO. These are ranked in order of similarity in Table 11. It is important to note that a similar BLAST search using Lipoxygenase enzymes failed to detect homologues. From the BLAST search, *Pxt* was identified as being homologous to both COX and

MPO. This was anticipated as previous studies had suggested that *Pxt* was a *Drosophila* COX homologue (Tootle and Spradling, 2008). We assayed knockouts of each potential homologue to establish whether they could reduce tumour incidence like COX and MPO inhibitors.

Table 11. MPO, COX-1 and COX-2 *Drosophila* homologues ranked in order of similarity

| COX1 | | Cox-2 | | MPO | |
|-----------------|----------|-----------------|----------|-----------------|-----------|
| Candidate | E value | Candidate | E value | Candidate | E value |
| <i>CG4009</i> | 2.00E-11 | <i>CG10211</i> | 2.00E-15 | <i>Pxn</i> | 9.00E-162 |
| <i>Pxt</i> | 1.00E-10 | <i>Irc</i> | 9.00E-12 | <i>CG10211</i> | 1.00E-101 |
| <i>CG10211</i> | 2.00E-09 | <i>Pxt</i> | 9.00E-12 | <i>cardinal</i> | 7.00E-81 |
| <i>Pxd</i> | 1.00E-07 | <i>CG4009</i> | 4.00E-11 | <i>Pxt</i> | 5.00E-77 |
| <i>Irc</i> | 1.00E-07 | <i>cardinal</i> | 2.00E-09 | <i>CG4009</i> | 3.00E-72 |
| <i>Pxn</i> | 1.00E-07 | <i>Pxd</i> | 1.00E-08 | <i>CG4009</i> | 5.00E-58 |
| <i>cardinal</i> | 1.00E-07 | <i>Pxn</i> | 2.00E-08 | <i>dDuox</i> | 1.00E-49 |
| <i>dDuox</i> | 2.00E-05 | <i>dDuox</i> | 1.00E-06 | <i>Irc</i> | 3.00E-29 |

Four out of the eight potential MPO/COX homologues significantly reduced tumour incidence compared to the control. The *dDuox* deficiency *Df(2L)BSC692* suppressed tumour number by 19.5% (Figure 11A). This was on par with the candidate *Pxt*, where the mutant *Pxt*^{f05258} reduced tumour incidence by 25% (Figure 11A). The effectiveness of the *Pxt* insertion at reducing expression was clarified using RT-PCR that confirmed *Pxt*^{f05258} reduces *Pxt* transcript levels over 1000 fold (Figure 11B). Another candidate *Irc* successfully reduced tumour number. The *Irc* mutant *Irc*^{MB11278} reduced the incidence of inflammatory tumours by 42.2% (Figure 11A). We also clarified the reduction in levels of this insertion using RT-PCR that confirmed *Irc*^{MB11278} reduces *Irc* transcript levels by over 10 fold (Figure 11B). The final candidate to significantly reduce the inflammatory phenotype was *Pxn*. The *Pxn*

mutant *Df(3L)BSC119* gave the largest reduction of tumour incidence of 53.4% (Figure 11A). There is no specific mutation available that removes *Pxn* alone. To give strength to the suppression we observed being a result of *Pxn* deletion, we used another deficiency *Df(3L)Exel160901* that overlaps the *Pxn* deficiency, removing several genes the *Pxn* deficiency does but not *Pxn* itself. *Df(3L)Exel160901* failed to significantly reduce tumour incidence suggesting *Pxn* is the interactor (Figure 11A). Mutants of *cardinal*, *CG4009*, *Pxd* and *CG10211* also failed to significantly reduce tumour incidence.

Interestingly, using alignment of *Pxn*, *Irc*, *Pxt* and *dDuox* to human COX-1 using the program ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), we identified conserved residues that are fundamental to cyclooxygenase activity. Tyr385 was found to be Phe385 in all four proteins. For *Pxn*, *Irc* and *Pxt*, Val349 was found to be Leu349 in all three proteins, and finally Arg120 was conserved in *Pxt* and found to be Glu120 in *Pxn* and *Irc*. This adds strength to the homologues being potential candidates of COX. We carried forward *Pxn*, *Pxt* and *Irc* due to the significant reduction in tumour incidence and conserved fundamental residues in COX activity. Although *dDuox* showed a reduction in tumour incidence, it was not as significant as the other candidates, and it lacked key conserved residues and its sequence similarity ranked low in all homologues.

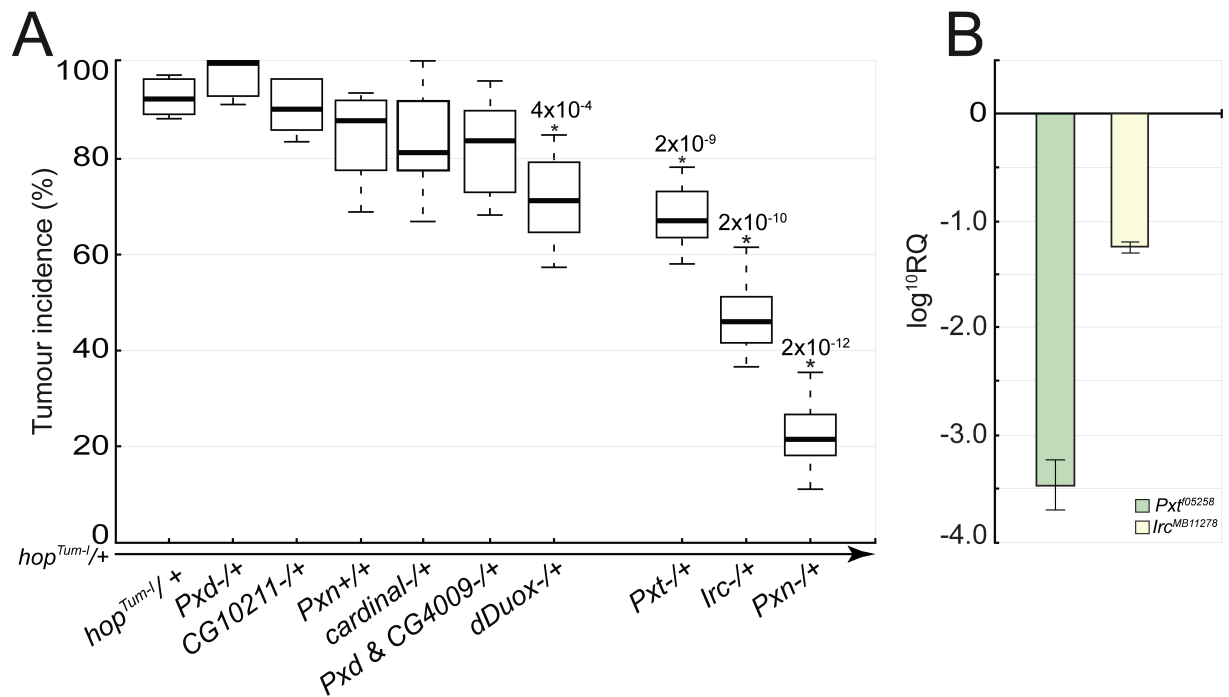


Figure 11. Effect of *Drosophila* MPO/COX homologue mutations on tumour incidence

Heterozygote mutant/*hop^{Tum}* females for each MPO/COX homologue were assayed for their effect on tumour incidence (%). Flies were incubated at 27°C to assay for suppression of tumours. **(A)** Eight potential candidate homologues were assayed. *Pxd*⁻: *Pxd^{LL00313}*, *CG10211*⁻: *Df(2L)BSC149*, *Pxn*⁺ (Used as an overlap deficiency to verify *Pxn* deleted effects in *Pxn*⁻): *Df(3L)Exel16091*, *cardinal*⁻: *Df(3R)Exel6192*, *Pxd* and *CG4009*⁻: *Df(3R)Exel8165*, *dDuox*⁻: *Df(2L)C144*, *Pxt*⁻: *Pxt^{f05258}*, *Irc*⁻: *Irc^{MB11278}*, and *Pxn*⁻: *Df(3L)BSC119*. * Indicates those values that are statistically significant having a P-value less than 0.001. **(B)** Real time PCR was used to verify expression *Irc^{MB11278}* and *Pxt^{f05258}* deletion. Transcript expression levels were normalised to *RpL32*. Only the strongest allele is shown for each candidate, details can be found in Table 1.

3.1.5 Discussion

In this work we have shown that supplementing with essential fatty acids, linoleic acid and α -linolenic acid, could affect the production of inflammatory mediators. We have identified a likely biosynthetic pathway by which *Drosophila* produce free fatty acids such as AA. This relies on the *Drosophila* homologues of sPLA2 and PLC, *small wing*, *norpA* and *Plc21C*. We have shown that we can reduce inflammatory incidence with the use of COX-1, COX-2 and MPO inhibitors demonstrating that we are able to inhibit MPO/COX-like activity that prevents the synthesis of inflammatory lipid mediators like prostaglandins. We have identified three putative MPO/COX homologues; *Pxt*, *Irc* and *Pxn*. Our data suggest that *Drosophila* utilise a fatty acid precursor to modulate inflammation through either MPO or COX activity.

3.1.5.1 The effect of dietary lipids in *Drosophila* inflammation.

Our data showed that supplementation with omega-6 fatty acid linoleic acid significantly increased tumour incidence. This is consistent with data from nutritional studies that show increase in dietary omega-6 fatty acid is associated with the onset and progression of inflammatory diseases (Hibbeln et al., 2006). This in part is because an increase in omega-6 fatty acid results in an increase of pro-inflammatory mediators (like AA), providing an ideal inflammatory environment (Mohrhauer and Holman, 1963). In contrast, supplementation of omega-3 fatty acid α -linolenic acid reduced inflammatory tumours. This agrees well with the known anti-inflammatory properties of omega-3 fatty acids in other systems, and suggests that *Drosophila* metabolise α -linolenic acid into the anti-inflammatory mediators EPA and DHA.

Interestingly, we detected competing actions of omega-3 and omega-6 fatty acid, where supplementation with omega-3 fatty acid reversed the pro-inflammatory effect of omega-6 fatty acids. It is possible that *Drosophila* metabolise α -linolenic acid more efficiently than linoleic acid, as when safflower oil and flaxseed oil were both

supplemented to 0.15% tumour incidence was still reduced, to the level that occurs when flaxseed oil alone is supplemented to the media. It is possible that the fly is able to beneficially shift to utilising α -linolenic acid, as it would be advantageous to *Drosophila* to produce anti-inflammatory mediators to resolve the inflammation induced by the *hop*^{Tum} tumour model, rather than potentiate the effects of this model further by utilising linoleic acid in producing pro-inflammatory mediators.

Our data suggests that only polyunsaturated fatty acids affects inflammation in our model. Supplementation with saturated fatty acid failed to have any significant effect on the tumour incidence, unlike in other systems where saturated fat is known to exacerbate chronic inflammatory diseases (Kennedy et al., 2009). Equally, cholesterol which has a strong association with cardiovascular diseases like atherosclerosis (Libby et al., 2000), did not greatly enhance tumours. It is possible that pro-inflammatory activities of cholesterol are balanced by functions in tissue repair that may have aided the repair to the damage caused in our inflammatory model. Nevertheless, this data potentially provides evidence that *Drosophila* exclusively utilise polyunsaturated fatty acids to synthesise pro- and anti-inflammatory lipid modulators.

3.1.5.2 Biosynthetic pathways that result in liberation of free fatty acid

We have shown that supplementation of key precursors to inflammatory mediators, omega-3 and -6 fatty acids affects inflammatory tumour number in our model. Key to this is the availability of the precursors to be utilised by enzymes like COX and MPO to produce the pro- and anti-inflammatory mediators. In mammals the principal route to fatty acid liberation such as AA, is via cytoplasmic PLA2-mediated hydrolysis of PIP₂. Our data indicate that *Drosophila* lack functional cytoplasmic PLA2 but do have

secretory sPLA2. Knockout of the *Drosophila* sPLA2 significantly reduce tumour number.

We have shown that reduction in the levels of *Drosophila* PLC homologues also decrease tumour incidence substantially. PLC represents another biosynthetic route for AA (free fatty acid). PLC activation results in the production of diacylglycerol (DAG), these can be hydrolysed by DAG lipase into monoacylglycerol and AA. The three known *Drosophila* PLC homologues small wing, *Plc21C*, and *norpA* significantly reduced tumour incidence. *Plc21C* and *norpA* mutants had the strongest suppression on inflammatory tumour number, even stronger than that observed with sPLA2. To clarify further whether PLC functions as a source of AA synthesis, we have knocked out components of this pathway. As shown in Figure 9A, in *Drosophila*, synthesis of PIP₂ is regulated by phosphatidylinositol 4-phosphate 5 Kinase ((PIPK5) *skittles*). *Gaq* activates PLC (either *norpA* or *Plc21C*), which hydrolyses PIP₂ to produce DAG and IP₃. In turn, DAG is then hydrolysed by *inaE* (DAG lipase) into monoacylglycerol and AA. *rdgA* (DAG kinase) mediates the conversion of DAG to phosphatidic acid potentially reducing the production of AA. We have shown that *skittles*, *Gaq*, and *inaE* mutants suppress inflammatory tumours, whilst *rdgA* mutants increase inflammatory tumour incidence as expected.

We further clarified that the tumour reduction seen with these pathway components was due to reduced production of free fatty acid and not through other signalling pathways mediated through PLC. During hydrolysis of DAG by PLC, IP₃ is produced which when bound to its receptor regulates calcium signalling. Mutants in *Itp-r83A* (Inositol 1, 4, 5-tris-phosphate receptor), the *Drosophila* receptor for IP₃ failed to suppress tumour incidence but resulted in an enhancement of the phenotype. It is possible that calcium signalling may not pertain pro-inflammatory effects, but aids in the prevention and resolution of inflammation.

IP3 can further be processed by kinases to produce other inositol signalling molecules. IPK1 and IPK2 are involved in phosphorylating IP3 to produce IP5 and IP6. Mutants in *Drosophila* *lpk1* and *lpk2* showed significant reduction in tumour incidence, more so with *lpk2*, which produces IP6. The actions of IP5 and IP6 are not well characterised, but they have been shown to play an active role in regulating gene transcription and chromatin remodelling (Steger et al., 2003; Shen et al., 2003). Another signal transduction pathway of PLC is through activation of PKCs via DAG or calcium. We assayed four *Drosophila* PKC homologue mutants, *inaC*, *aPKC*, *Pkc53E* and *Pkc98E* that all sufficiently reduced tumour number, but were not as effective as mutants in IPKs. PKCs have an array of functions, so it is not surprising that they are able to reduce tumour incidence. It is important to add that although assaying for pathways involved in liberating free fatty acid, it is possible that particular knockouts can be exerting their effects on JAK/STAT signalling the model relies on. For example, the tumour incidence seen with IPK mutants could be the result of inhibition of STAT binding to its target and therefore lessens the phenotype. The stronger suppression seen with PLC mutants compared to sPLA2 could therefore be due to a combination of preventing free fatty acid liberation as well as inhibition of downstream signalling pathways that branch from PLC activation.

Overall, our data suggest that both sPLA2 and the PLC homologues play a vital role in the availability of free fatty acid such as AA. Though the functional existence of this pathway resulting in production of free fatty acid is still highly speculated (Stark et al., 1993). Our data suggests otherwise.

3.1.5.3 Suppression of tumour incidence using MPO and COX inhibitors

Using a non-selective COX inhibitor aspirin, as well as a COX-1 specific inhibitor SC-560, a COX-2 specific inhibitor NS-398 and an MPO specific inhibitor MPO inhibitor-

1, we have established that MPO/COX activity is present in *Drosophila*. All inhibitors suppressed inflammatory tumours significantly.

Interestingly, for aspirin and SC-560 the experimentally determined IC₅₀s for tumour suppression were closer to the IC₅₀ for COX-1 (Table 10). In contrast, for NS-398 the experimentally determined IC₅₀ for tumour suppression was closer to the IC₅₀ for COX-2. These data suggest that *Drosophila* COX enzymes are intermediate between the COX-1 and COX-2 which share similar residues involved in the inhibitor action. In terms of MPO, the experimental IC₅₀ also was lower than the published suggesting that peroxidase activity is present. What is striking is that all experimental IC₅₀s are lower than the published values. This could be due to species differences in the IC₅₀ of each drug or that published IC₅₀ values were calculated using isolated protein in vitro, whereas our results are generated using drugs that are supplemented to media, are ingested by larvae and potentially could be concentrated in these animals. Strengthening the argument that COX activity is present and can be inhibited is the presence of the Val349 residue, which is conserved in the candidate MPO/COX homologues identified. Val349 is known to be acetylated by aspirin and is involved in the inhibition of COX (Schneider et al., 2002)

3.1.5.4 Identification of candidate MPO/COX homologues

Using our tumour suppressor screen we have been able to exclude *Pxd*, *CG10211*, *cardinal* and *CG4009* as having either MPO or COX function on the basis that they did not affect tumour incidence in *Drosophila*. This was a surprise for *CG10211* as it was ranked most similar for both COX isoforms and MPO compared to the other candidates (Table 11). However, our data indicate that *dDuox*, *Pxt*, *Irc* and *Pxn* have MPO/COX activity. We can potentially assign MPO/COX function to *Pxt* and *Irc* due to the availability of gene specific insertions, which has been clarified using RT-PCR. The lack of specific mutations affecting only *dDuox* and *Pxn* preclude unambiguous

assignment of MPO/COX function to them. Though we have attained some clarity by using another deficiency that removes over half the genes affected in *Df(3L)BSC119*, but without affecting *Pxn*, and was observed to have no effect on tumour incidence. Currently no specific mutations exist for *Pxn*. However, the presence of key conserved residues involved in COX activity like Tyr385 are present in all four candidates strengthening their positions as candidates for possessing COX activity. Though dDuox lacks other residues important for cyclooxygenase activity. We discarded dDuox as a potential candidate because although dDuox deletions reduced tumour incidence, it was not as significant as the other candidates. Its sequence similarity to mammalian COX and MPO was also ranked low. dDuox has been shown to be involved in embryo wound healing that is reliant on calcium and hydrogen peroxide signalling (Razzell et al., 2013). The reduction in tumour incidence we observed could be due to calcium signalling dysfunction, though it is possible that dDuox is capable of lipid mediator production.

Given the extensive evidence suggesting an inflammatory lipid mediator pathway is present in flies using an indirect assay, it is paramount to biochemically identify whether such molecule are endogenously present in *Drosophila*. By using LC-MS we can identify the presence of AA and prostaglandins, and determine the actual lipid mediators *Drosophila* utilise in inflammatory processes.

3.2 Lipidomic analysis to identify lipid mediators and characterisation of those mediators identified

The *in vivo* tumour assay highlighted the involvement of pro and anti-inflammatory lipid mediators in regulating inflammation. The assay provided an indirect assessment of the existence and function of these mediators. However, it was paramount to identify these mediators using a refined specific detection method. Liquid chromatography–Mass spectrometry (LC-MS) provides a sensitive and accurate method to identify endogenous lipid mediators (Massey and Nicolaou, 2013). We therefore used LC-MS to characterise the lipid mediators involved in inflammation in *Drosophila*.

3.2.1 Identifying lipid mediators and their precursors in *Drosophila* using LC-MS

We conducted a whole fatty acid analysis using LC-MS to identify the presence of the key precursors of inflammatory mediators that include AA, EPA and DHA. By analysing *hop*^{Tum} larvae grown on media supplemented with 0.15% linoleic acid it was expected to drive production of pro-inflammatory mediators and therefore aid in the detection. Larvae were analysed in batches of 100 mg and 200 mg to confidently detect fatty acids that are of lower abundance. LC-MS whole fatty acid analysis revealed that both linoleic acid (C18:2n-6c highlighted in red) and α -linolenic acid (C18:3n-3; highlighted in green) were present, albeit linoleic acid is more abundant being 7% of total fatty acid (Table 12). AA (C20:4n-6 highlighted in yellow) failed to be detected (Table 12). EPA (C20:5n-3 highlighted in purple) and DHA (C22:6n-3 highlighted in blue) also were not detected (Table 12). We observed that the bulk of fatty acids present in *Drosophila* exhibit a carbon chain length of C18 and below.

Table 12. LC-MS whole fatty acid analysis showing % of total fatty acid

| Sample Weight (mg) | 100 | 200 |
|--------------------|------------|------------|
| Fatty Acid | % Total FA | % Total FA |
| C4:0 | 0 | 0 |
| C6:0 | 0 | 0 |
| C8:0 | 0 | 0 |
| C10:0 | 0.01 | 0.02 |
| C11:0 | 0.01 | 0.01 |
| C12:0 | 2.44 | 2.57 |
| C13:0 | 0 | 0 |
| C14:0 | 22.49 | 20.99 |
| C14:1 | 1.50 | 1.73 |
| C15:0 | 0.13 | 0.02 |
| C15:1 | 0 | 0 |
| C16:0 | 23.48 | 22.68 |
| C16:1 | 19.86 | 23.59 |
| C17:0 | 0.12 | 0 |
| C17:1 | 0.19 | 0.13 |
| C18:0 | 2.50 | 1.85 |
| C18:1n-9t | 0 | 0 |
| C18:1n-9c | 19.40 | 19.16 |
| C18:1n-7 | 0 | 0 |
| C18:2n-6t | 0 | 0 |
| C18:2n-6c | 7.23 | 6.77 |
| C18:3n-6 | 0 | 0 |
| C18:3n-3 | 0.19 | 0.21 |
| C20:0 | 0.32 | 0.19 |
| C20:1n-9 | 0.01 | 0.03 |
| C20:2 | 0 | 0 |
| C20:3n-6 | 0 | 0 |
| C20:4n-6 | 0.01 | 0.01 |
| C20:3n-3 | 0 | 0 |
| C22:0 | 0.08 | 0.03 |
| C22:1n-9 | 0.02 | 0.01 |
| C20:5n-3 | 0 | 0 |
| C23:0 | 0 | 0 |
| C22:2 | 0 | 0 |
| C24:0 | 0.01 | 0 |
| C24:1 | 0 | 0 |
| C22:5n-3 | 0 | 0 |
| C22:6n-3 | 0 | 0 |

The failure to detect significant levels of AA suggested that prostaglandins would be absent. To confirm this, we used LC-MS to detect PGE₂, PGF_{2α} and PGD₂ from 200 mg larvae samples. Known PG standards were ionised during MS resulting in a fragment ion distribution. Three fragment ions specific to each PG were chosen, and multiple reaction monitoring mode was used to identify each specific fragment ion in the sample.

Using PGE₂ specific mass/charge fragment ions 271, 315 and 333 at a retention time of 4.85 min, failed to detect PGE₂ in both larvae raised on media supplemented with linoleic acid (omega-6 fatty acid) and α-linolenic acid (omega-3 fatty acid) (Figure 12). This was also true for standard media, which was used as a control to establish if PGs were present in the *Drosophila* diet. PGD₂ was not directly assayed, but due to its similar ion fragmentation pattern to PGE₂, where both PGs share a fragment ion that has a retention time of 5.75 min, we could assay PGD₂ and failed to detect PGD₂ in the extracts. For PGF_{2α}, the fragment ions 193, 247 and 309 at a retention time of 3.96 min were used. PGF_{2α} is very unstable and is quickly converted to 8-iso-PGF_{2α} having a retention time of 3.44 min. Both PGF_{2α} forms were also not detected in larvae grown on supplemented media or in control media (Figure 13).

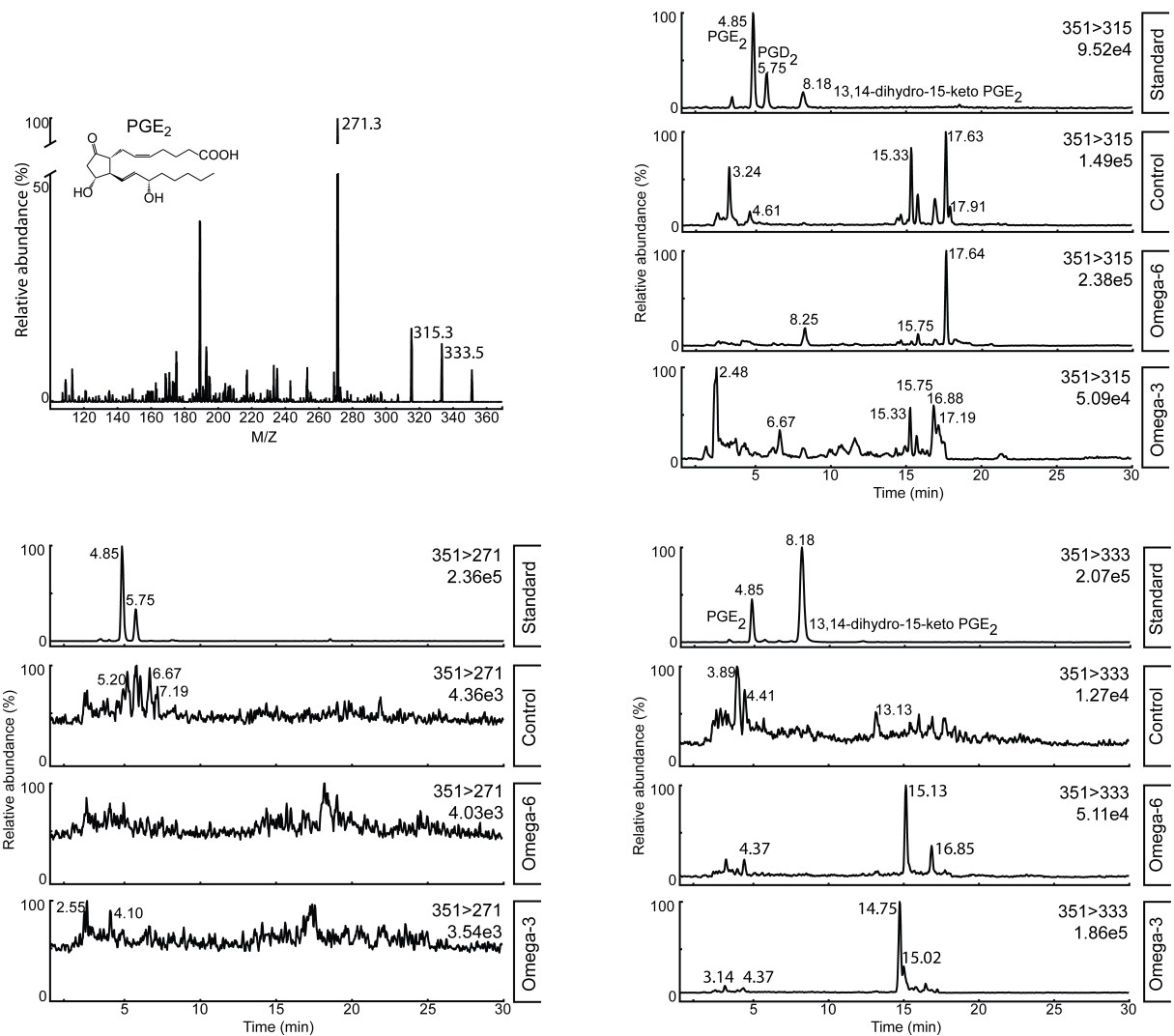


Figure 12. LC-MS detection of PGE₂

A PGE₂ standard of relative atomic mass 351 elicits a fragment ion spectrum (Top left) that shows the three specific fragment ions that had an m/z ratio of 271, 315 and 333. LC-MS using MRM was used to detect PGE₂ fragments at a retention time of 4.85 min as shown in the standard chromatogram for each fragment ion. Standard *Drosophila* media was used as a control to determine PG presence in the food. *hop*^{Tum} larvae raised on media supplemented with either 0.15% omega-3 fatty acid or omega-6 fatty acid were assayed for the presence of PGE₂. PGE₂ and PGD₂ share a similar fragment ion at a retention time of 5.75 min.

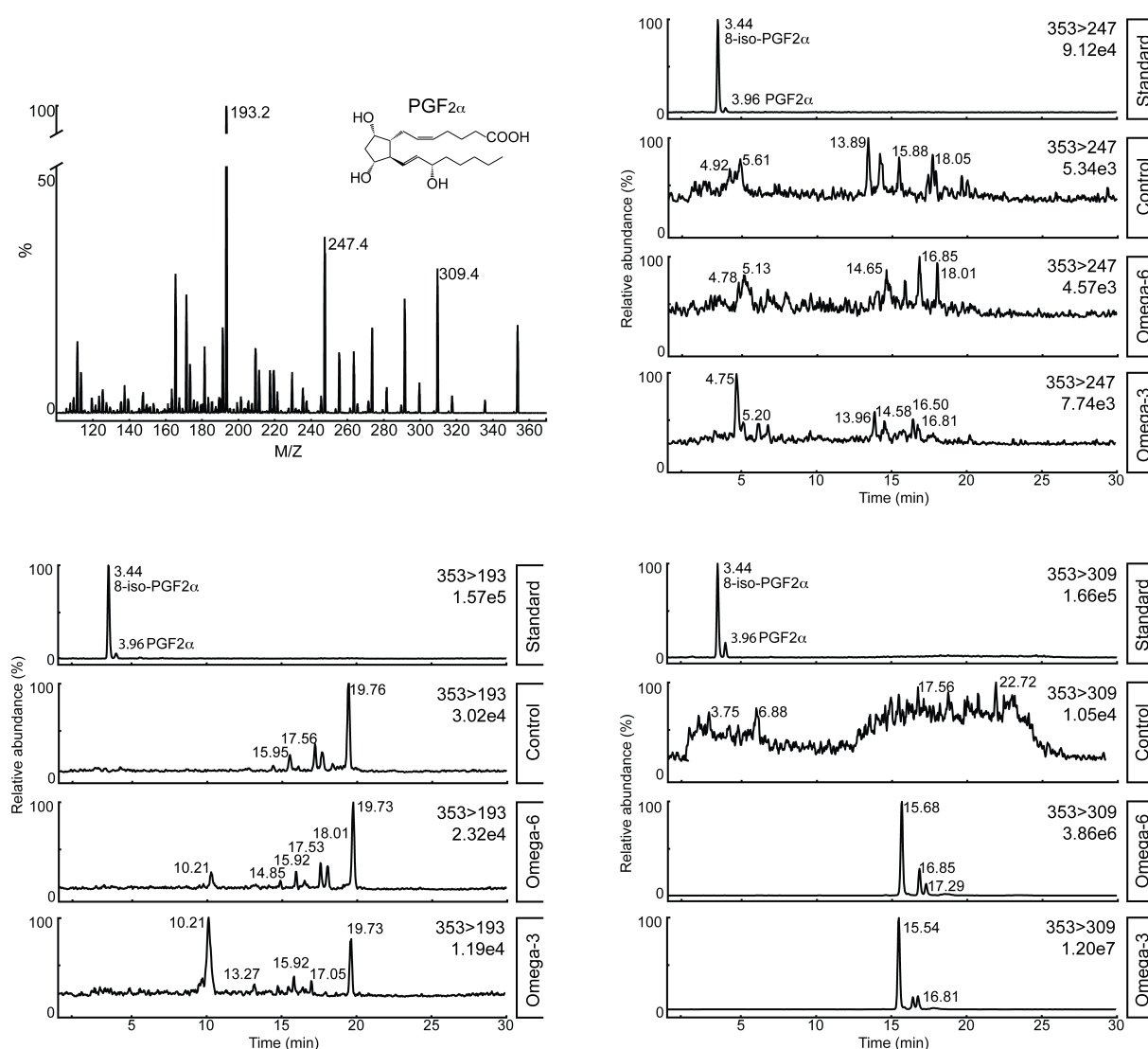


Figure 13. LC-MS detection of PGF_{2α}.

A PGF_{2α} standard of relative atomic mass 353 elicits a fragment ion spectrum (Top left) that shows the three specific fragment ions that had an m/z ratio of 193, 247 and 309. LC-MS using MRM was used to detect PGF_{2α} fragments at a retention time of 3.96 min as shown in the standard chromatogram for each fragment ion. PGF_{2α} is unstable and is quickly converted to 8-iso-PGF_{2α} that has a retention time of 3.44 min. Standard *Drosophila* media was used as a control to determine PG presence in the food. *hop^{Tum}* larvae raised on media supplemented with either 0.15% omega-3 fatty acid or omega-6 fatty acid were assayed for the presence of PGF_{2α}. PGE₂ and PGD₂ share a similar fragment ion at a retention time of 5.75 min.

As both C20 fatty acids and C20 derived inflammatory mediators were absent, we examined whether lipid mediators that can be synthesised from C18 fatty acids were present. From the literature we identified hydroxyl-octadecadienoic acids HODEs as being direct products of linoleic acid and having immuno-modulator effects (Vangaveti et al., 2010). The LC-MS data from the PG detection was re-analysed using a reconstructed-ion chromatogram, whereby a known mass-charge-ratio value was used in a single monitoring reaction (SMR) mode to identify the molecules. We analysed for the presence of both 9- and 13-HODE. We were able to detect 9-HODE using a fragment ion of m/z 171 and a retention time of 15.18 min (Figure 14A). Similarly for 13-HODE, using a fragment ion of m/s 195 and a retention time of 14.98 min revealed, it too was present (Figure 14B).

In using the HODE spectra, we calculated the abundance of 9- and 13-HODE in w^{1118} (control) and hop^{Tum} strains raised on standard media, and media supplemented with 0.15% omega-3 fatty acid or 0.15% omega-6 fatty acid. We observed the w^{1118} strain to have a higher abundance of both 9- and 13-HODE compared to the hop^{Tum} strain. It is apparent that supplementation with omega-3 and -6 fatty acid affects the abundance of HODEs in each strain. In w^{1118} larvae, supplementation with omega-3 fatty acid reduced the level of 9-HODE by 17.6%, but increased 13-HODE by 21.2% compared to the control (Figure 14C). Whereas supplementation with omega-6 fatty acid resulted in an increase of 47% for 9-HODE and a 57.8% increase in 13-HODE. In terms of the hop^{Tum} strain, omega-6 fatty acid supplemented larvae showed an increase of 22.4% for 9-HODE and an increase of 42.6% for 13-HODE (Figure 14D). While omega-3 fatty acid supplementation showed an increase of 12.5% for 13-HODE, only an increase of 3.4% was seen for 9-HODE.

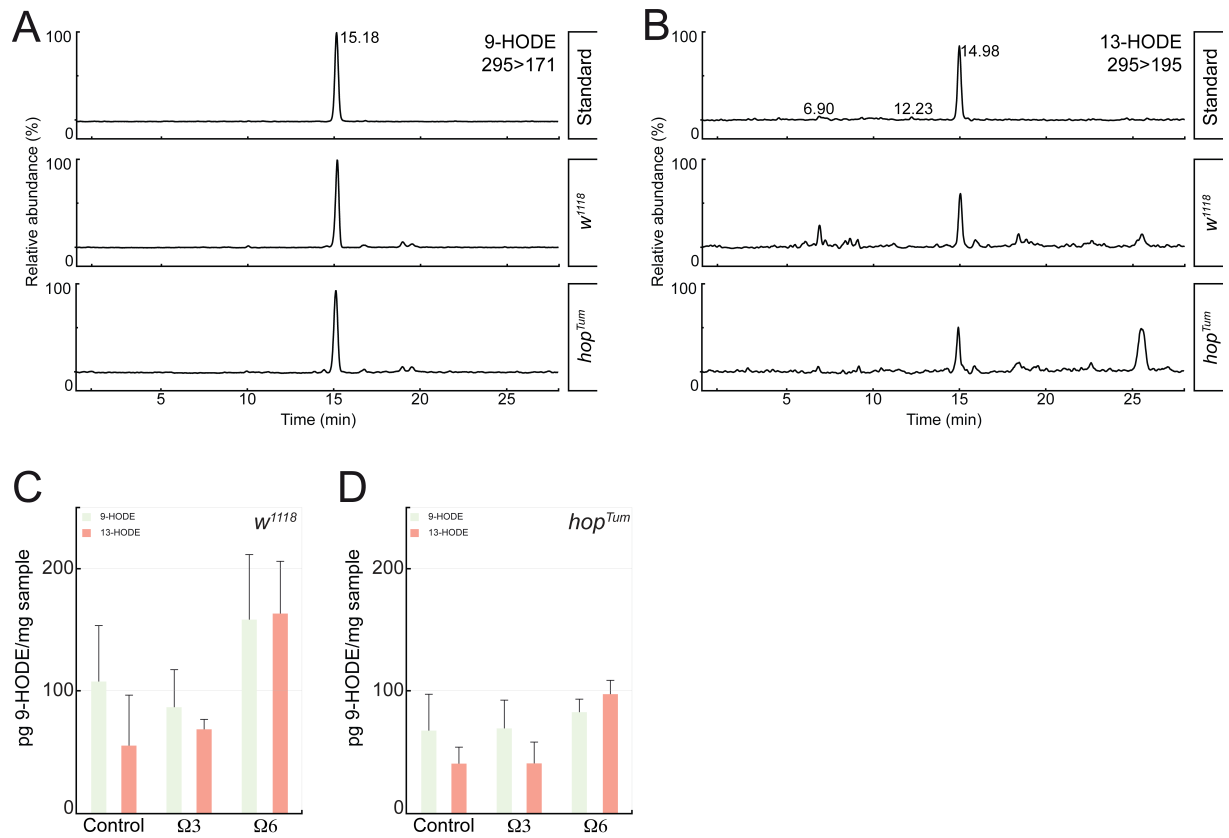


Figure 14. LC-MS identification of 9- and 13-HODE

LC-MS using single monitoring reaction detected 9- and 13-HODE in wild type *w¹¹¹⁸* larvae, and *hop^{Tum}* larvae. **(A)** 9-HODE was identified using a fragment ion of a relative atomic mass 171 at a retention time of 15.18. **(B)** 13-HODE was identified using a fragment ion of a relative atomic mass 195 min at a retention time of 14.98 min. The relative amount of both 9- and 13-HODE was calculated for *w¹¹¹⁸* **(C)** and *hop^{Tum}* **(D)** by measuring the area under the chromatogram peaks. n=3

We next tested whether HODEs were produced enzymatically. For HODEs, two enantiomers exist; an R and an S form. Typically S enantiomers of compounds are produced enzymatically while the R enantiomers are usually formed by non-enzymatic activity. LC-MS chiral analysis was conducted using *w¹¹¹⁸* and *hop^{Tum}* larvae extracts. We observed that *w¹¹¹⁸* and *hop^{Tum}* larvae have similar levels of R and S form of HODES in control and omega-3 supplemented (Figure 15). The 9-S-HODE enantiomer was the predominant form in all media treatments. Lower levels of 9-R-HODE, 13-R-HODE, and 13-S-HODE were detected.

Interestingly, for *w¹¹¹⁸* larvae raised on omega-6 fatty acid supplemented media, there is a dramatic increase in the production of S and R forms of 9- and 13-HODE (Figure 15A). The same is seen in *hop^{Tum}* larvae, but the levels seen in *w¹¹¹⁸* surpass the levels observed in *hop^{Tum}* larvae (Figure 15B). In terms of the R-enantiomer, it too increases with omega-6 supplementation for both 9- and 13-HODE.

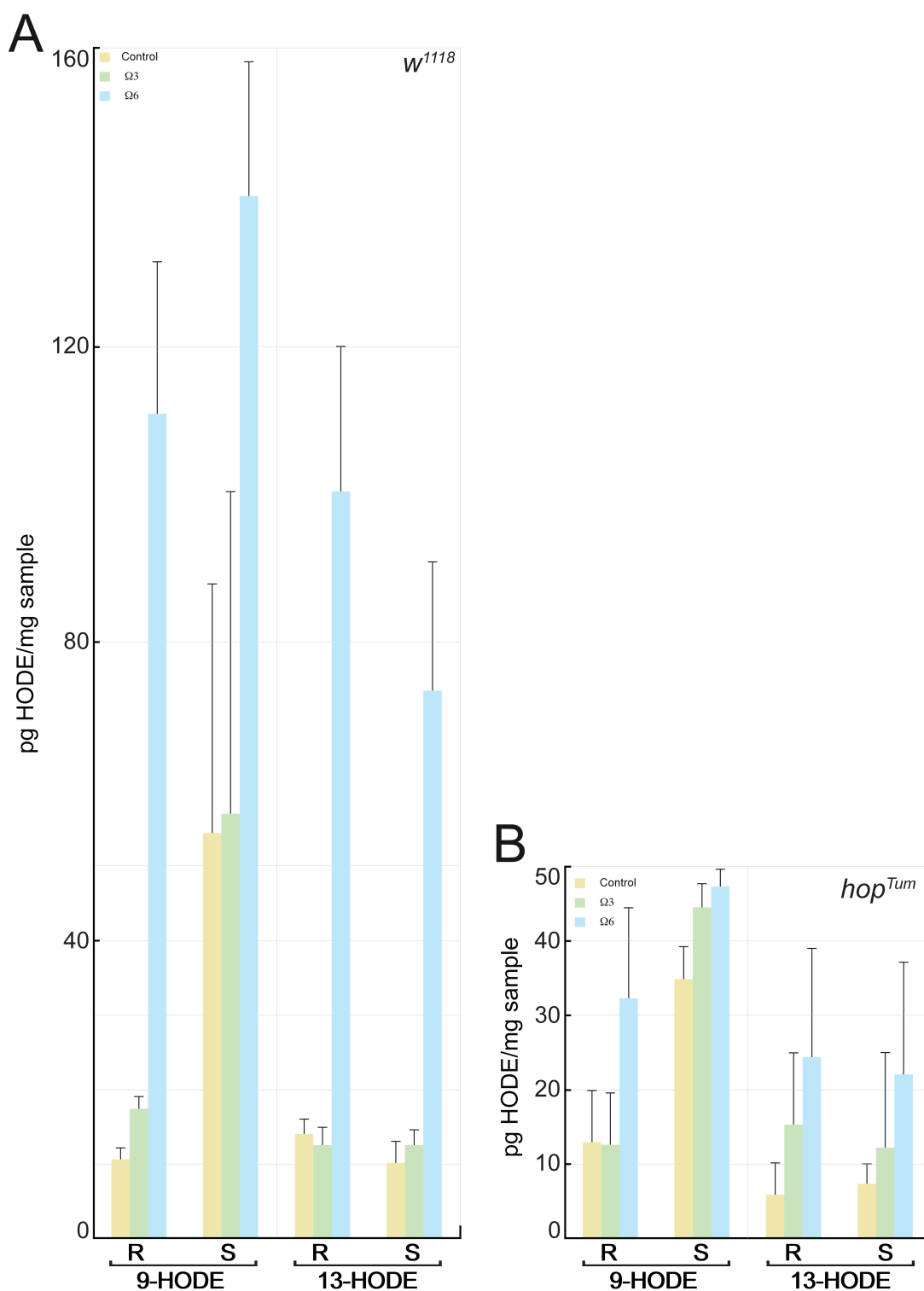


Figure 15. LC-MS chiral analysis of 9- and 13-HODE

9- and 13-HODE chiral enantiomers were separated using a LUX cellulose-1 3 μ M, 150 x 2.0 mm column. **(A)** *w¹¹¹⁸*, and **(B)** *hop^{Tum}* larvae were assayed for the presence of both the R and S form for each HODE. Chiral abundance is shown as HODE (pg)/Sample (mg). Larvae raised on standard media (control), omega-3 supplemented and omega-6 supplemented media were assayed. n=2

3.2.2 Characterisation of 9- and 13-HODE function

9- and 13-HODE have been implicated to have an immuno-modulatory role in mammalian systems. We sought to clarify whether the compounds have similar effects in *Drosophila*. We used a mixture of *in vivo* and *in vitro* assays to determine the role 9- and 13- HODE play in *Drosophila*. HODEs have well documented functions in atherosclerosis, being detected in plaques and implicated in the progression of the disease. HODEs have been indicated to play a role in the differentiation of macrophages into lipid laden foam cells. The *hop*^{Tum} strain has an abundance of large differentiated lamellocytes that show superficial similarities to foam cells, though it was unclear if they accumulated lipid.

To determine lipid content, haemocytes from control and *hop*^{Tum} larvae were stained with Oil Red O, which is a lysochrome diazo dye that specifically stains lipid that can be used to specifically shown lipid deposits (Koopman et al., 2001).

We observed Oil Red O staining of *hop*^{Tum} haemocytes was heavily stained by Oil Red O, showing that they are lipid laden (Figure 16). It was noticeable that haemocytes in the *hop*^{Tum} strain are larger than control haemocytes being 40 µm larger than *w*¹¹¹⁸ haemocytes. Larger haemocytes of the *hop*^{Tum} larvae contained more lipid than smaller haemocytes. Interestingly, Oil Red O staining of the *hop*^{Tum} melanotic tumours reveals that they contain extensive lipid deposits and are aggregates of lamellocytes and/or contain fat body. Conversely, Oil Red O staining of the fat body revealed *hop*^{Tum} larvae to have drastically diminished lipid storage compared to *w*¹¹¹⁸ that showed strong staining (Figure 16). The fat body is the equivalent of vertebrate adipose tissue and is important for a number of functions such as AMP production and insulin signalling.

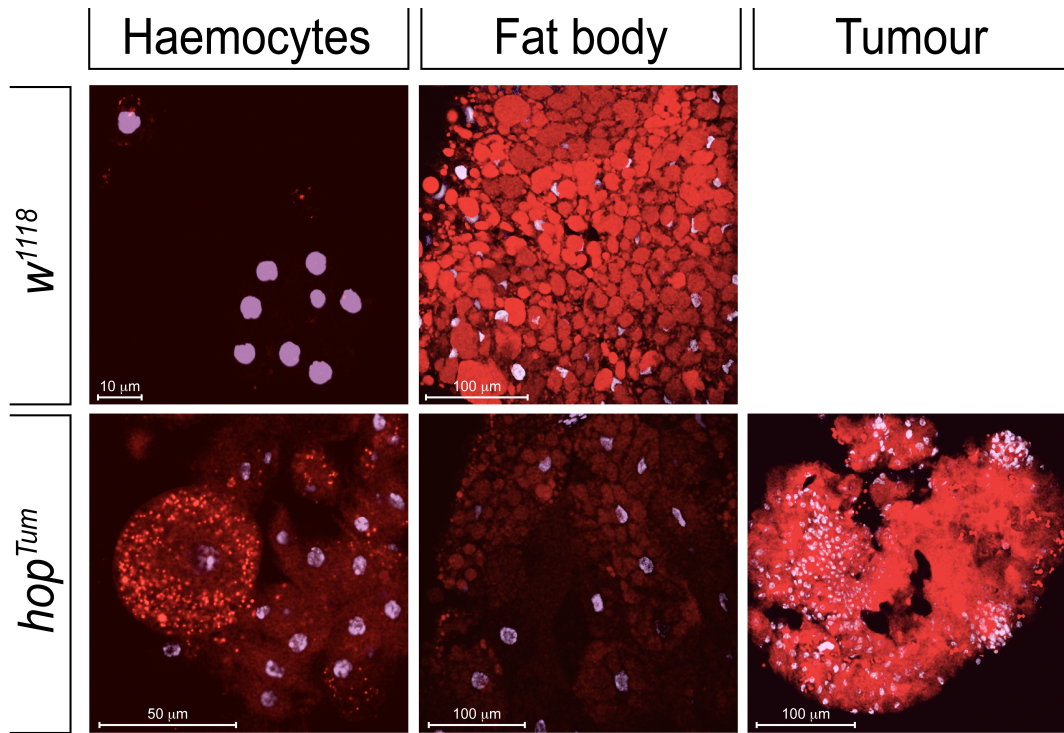


Figure 16. Oil Red O staining of *w¹¹¹⁸* and *hop^{Tum}* haemocytes and fat body

Oil Red O, a lysochrome diazo dye that specifically stains lipid was used to stain haemocytes and fat body tissue from *w¹¹¹⁸* and *hop^{Tum}* larvae. *hop^{Tum}* tumours were also stained. The scale bar for haemocytes is 50 μm , and 100 μm for fat body and melanotic tumours.

9- and 13-HODE have been shown to be ligands of the PPAR γ nuclear receptor that is involved in adipogenesis and activation of macrophages (Itoh et al., 2008; Vangaveti et al., 2010). We sought to identify a PPAR γ homologue in *Drosophila* using BLAST. Ecdysone-induced protein 75B (Eip75B) was shown to be the closest homologue of PPAR γ . Eip75B is a nuclear receptor that has largely been characterised for its involvement in the molt stages of *Drosophila* (Thummel, 2001). We used strains in which *Eip75b* is deleted and assayed the effects of these on the *hop^{Tum}* inflammatory model. Knockout of *Eip75b* reduced tumour incidence by 39.7% (Figure 17A). To validate whether 9- and/or 13-HODE could bind to *Eip75B* and modify transcriptional activity, we used a *Drosophila* strain in which the Eip75B

ligand-binding domain (LBD) was fused with GAL4. The strain background also contains UAS-GFP that would act as a reporter for GAL4. The GAL4-UAS system is widely used: UAS is an enhancer that contains binding sites for the GAL4 transcription factor. In this strain, although GAL4 can bind to UAS-GFP, transcription will not occur unless the Eip75B-LBD that is fused to GAL4 is activated by a ligand. Haemocytes heat-shocked to induce expression of Eip75B-LBD GAL4 were treated with 9- and 13-HODE and visualized for GFP-reporter expression. We observed no response to treatment with 9- or 13-HODE (Figure 17B). Interestingly, Diethylenetriamine NONOate (DETA), a NO donor also did not show any response compared to the control. DETA is a positive control as it has been shown NO binds to Eip75b allowing it to interact with its heterodimer partner DHR3 resulting in transcription (Reinking et al., 2005).

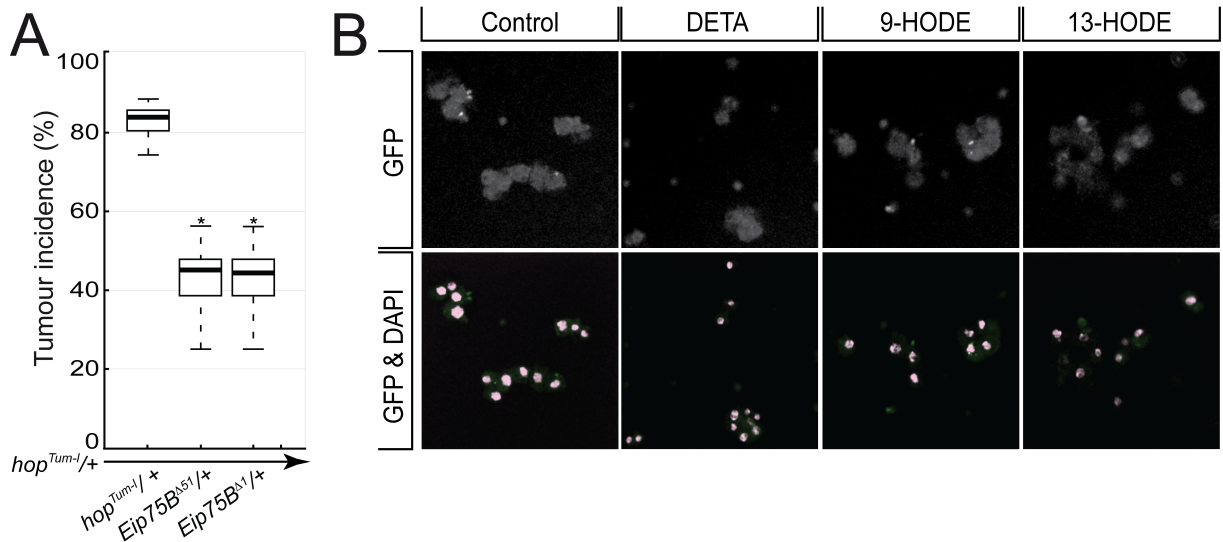


Figure 17. *Eip75B* tumour assay and Eip75B-LBD reporter assay

(A) The effect of *Eip75B* knockout mutants on inflammatory tumour incidence. An Eip75B-LBD-GAL4 fusion UAS-GFP reporter line was used to determine if 9- and 13-HODE were ligands of *Eip75B*. **(B)** Haemocytes were cultured in control media, media containing Diethylenetriamine NONOate (DETA), a NO donor that is a positive control for Eip75B activation, and 9- and 13-HODE. Nuclei were stained with DAPI and the reporter activity visualised using GFP.

To ascertain directly the affects of 9- and 13-HODE, we used *Drosophila* Schneider 2 cells (S2 cells) that are an embryo derived macrophage-like cell line, to assay the transcriptional effects of 9-HODE, 13-HODE, as well as omega-3 fatty acid, omega-6 fatty acid and a mixture of omega-3 and -6 fatty acid. An equal mix of omega-3 and -6 fatty acid was used to determine if the effects seen with omega-6 fatty acid could be suppressed in the presence of omega-3 fatty acid. The S2 cells were cultured for 48 hours in each condition and the RNA subsequently extracted to analyse transcript levels of known markers pertaining to a particular haemocyte cell lineage. Due to HODEs playing a role in macrophage differentiation, we expected to see upregulation of differentiation markers.

We traditionally used levels of *RpL32* as an endogenous control to normalise levels of expression of the transcripts. It was noted that although equimolar amounts of mRNA was used for input, *RpL32* levels varied dramatically after treatment, especially with fatty acids. We therefore analysed expression of two further housekeeping genes (Figure 18B). When transcript levels were normalised to α -*Tubulin* we observed in omega-6 fatty acid treated cells, a 25-fold upregulation of *RpL32*, a gene involved in translation, and a 9-fold upregulation of *Gapdh2*, a gene involved in glycolysis (Figure 18A). Omega-3 fatty acid treatment resulted in an upregulation between 1 and 4-fold for *RpL32* and *Gapdh2*. Treatment with 9- and 13-HODE had little affect on their transcript expression. *RpL32* and *Gapdh2* expression may be upregulated due to an increase in metabolic activity as the cells actively uptake the supplemented fatty acids which in turn requires energy, especially if protein synthesis is increased.

In terms of the differentiation markers, HODEs and omega fatty acid treatments had very little effect on the lamellocyte markers *myospheroid (mys)*, *Integrin Bv subunit*

(*Itgβv*), *cheerio* (*cher*) and *Integrin αPS4 subunit* (*ItgαPS4*) (Figure 18A). However, both 9- and 13-HODE, including omega-6 fatty acid treated cells markedly increased expression of the lamellocyte marker *ItgαPS5* by 9-fold for both HODEs and up to 25-fold for omega-6 fatty acid. Omega 3 fatty acid treatment showed little change in comparison. 9-HODE treatment compared to 13-HODE treatment was observed to upregulate both *Iz*, a crystal cell marker and *Hml*, a plasmatocyte marker (Figure 18A). Whereas both were able to result in an upregulation of *He* as did the omega treatments. It was noticeable that omega-3 fatty acid treatment had little affect on differentiation markers compared to the other treatments. It was also shown that omega-3 fatty acid was unable to counteract the effects of omega-6 fatty acid in all genes assayed.

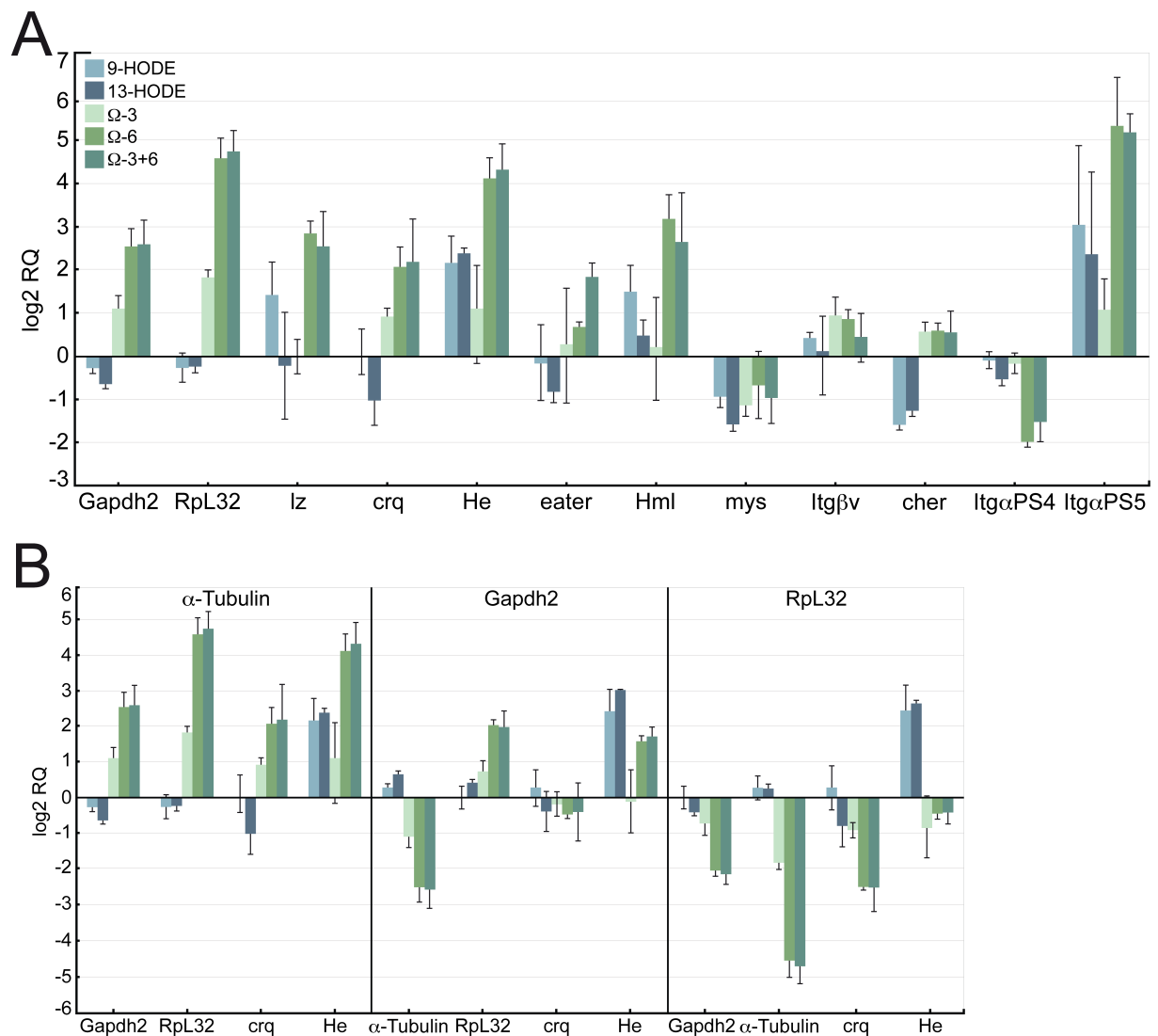


Figure 18. RT-PCR analysis of haemocyte differentiation makers in S2 cells treated with 9-HODE, 13-HODE, omega-3 and omega-6 fatty acid

S2 cells were cultured in standard media (control), 9-HODE, 13-HODE, omega-3 fatty acid, omega-6 fatty acid and an equal mix of omega-3 and -6 fatty acid for 48 hours to determine differences in transcript expression of known differentiation markers. **(B)** House keeping genes *α-Tubulin*, *RpL32* and *Gapdh2* were analysed as endogenous controls. **(A)** *α-Tubulin* expression in control cells was used to normalise transcript expression of the differentiation markers of crystal cells, plasmatocytes and lamellocytes. Details of the genes analysed are detailed in Table 4.

The RT-PCR analysis identified several targets of both HODEs and Omega fatty acids. It was fundamental to directly assay if these changes in mRNA transcripts were accompanied by expression in protein levels. Hemese (He), a transmembrane protein that is a marker of haemocyte lineage was markedly upregulated by all treatments and provided an ideal target. In using anti-He antibodies to stain for He, we expected to see stronger staining with S2 cells treated with omega fatty acids compared to the control, as RT-PCR analysis showed a marked upregulation of He. Oil Red O staining of these cells clarified if the cells were ingesting the supplemented fatty acids.

The data showed that with both omega-3 and omega-6 fatty acid treatment, S2 cells actively ingest the lipid and were shown to stain strongly with Oil Red O compared to the control, with lipid droplets clearly visible in the cell's cytoplasm (Figure 19A). All three of the omega fatty acid treatments also showed a clear enhanced staining for *He* compared to the control showing that upregulation of gene transcript of *He* results in increased protein expression of He (Figure 19B).

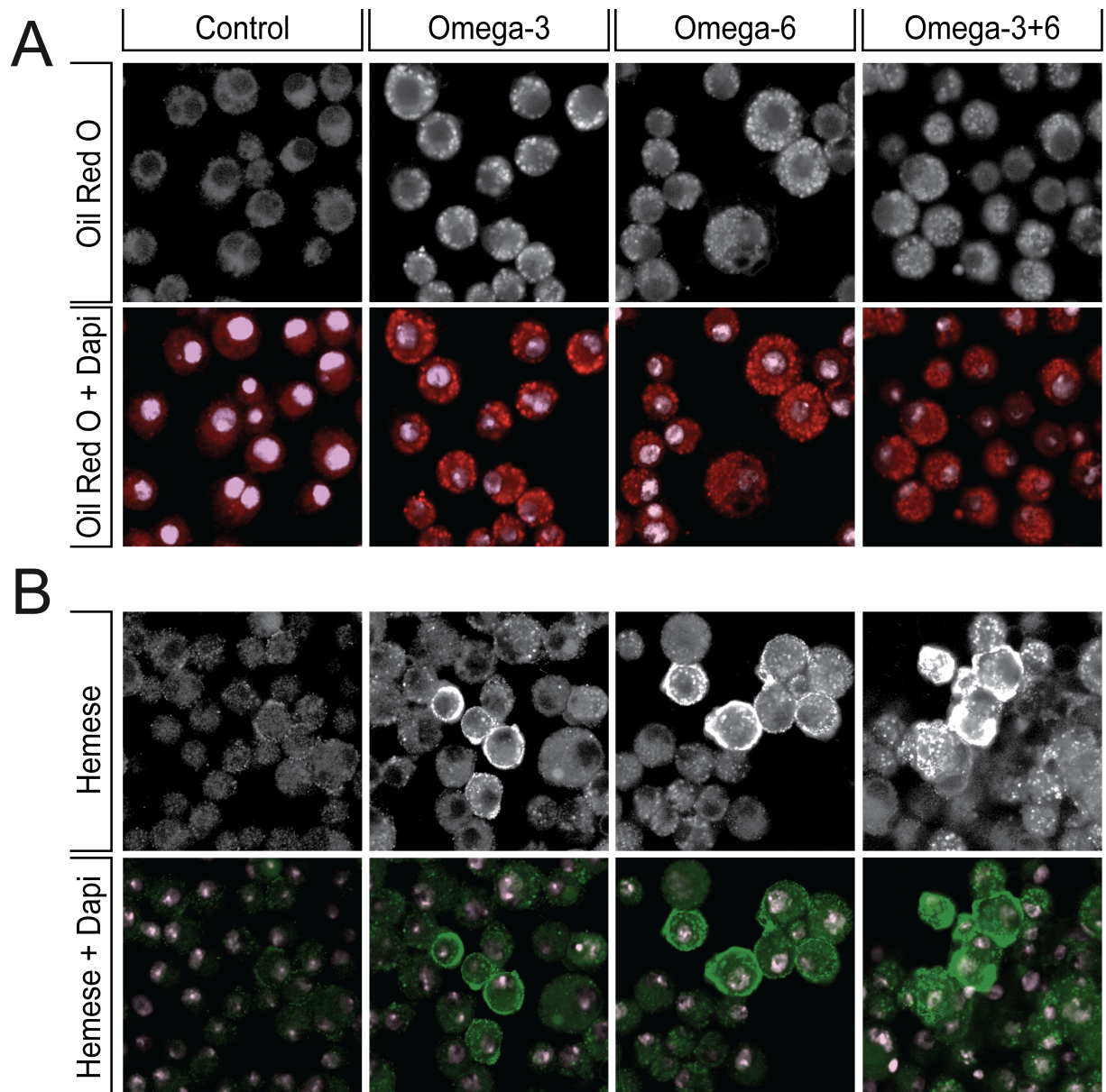


Figure 19. Oil Red O and Anti-He staining of omega fatty acid treated S2 cells

S2 cells were cultured in the presence of Omega-3 fatty acid, omega-6 fatty acid and an equal mix of omega-3 and -6 fatty acids for 48 hours and stained with **(A)** Oil Red O (red in merge) to visualise lipid uptake and **(B)** Anti-He (green in merge) to visualise expression of He. Dapi (purple in merge) was used to visualise nuclei.

3.2.3 Discussion

Consistent with other studies, we too have concluded that *Drosophila* do not synthesise AA. We purposely assayed larvae raised on 0.15% linoleic acid to ensure precursor availability was not a limitation and would therefore drive production of AA. It was observed that *Drosophila* can incorporate linoleic acid but are then unable to elongate beyond a C18 backbone. Moreover, flies lack EPA and DHA, two precursors formed from α -linolenic acid, adding strength to the theory that *Drosophila* cannot elongate beyond C18. α -linolenic acid was detected, albeit in lower quantities than seen with linoleic acid, this could be due to the supplemented diet containing little α -linolenic acid and therefore driving the incorporation of the more abundant linoleic acid. What is noticeable, are the trace amounts of fatty acids having a carbon backbone greater than C18 that were just under the limit of detection, including AA. It is possible that these are contaminants from the media. Another possibility is that *Drosophila* have been shown to convert supplemented C22 fatty acids into C20 fatty acids such as AA (Shen et al., 2010). Though they cannot synthesise AA, given the absence of $\Delta 5$ and $\Delta 6$ desaturases, they have the ability to convert longer chain fatty acids to C20 fatty acids.

The absence of AA was reflected by the absence of PGs. As with AA, there has been much speculation surrounding the existence of PGs in *Drosophila*. Several studies in *Drosophila* have suggested physiological effects that they have linked to PGs (Tootle and Spradling 2008; Groen et al., 2012), but all of the studies have involved indirect methods. We have directly assayed for PGs using LC-MS to conclusively reveal their absence in *Drosophila*. LC-MS provided a specific and accurate method to determine the presence of endogenous PGs. We assayed larvae raised on media supplemented with omega-3 fatty acid, and omega-6 fatty acid as well as standard media to ensure PGs were not present in the *Drosophila* diet. PGE₂ and PGF_{2 α} , both

failed to be detected in media alone, and larvae raised on either omega-3 or -6 fatty acid supplemented media. The absence of AA and PGs in *Drosophila* raised the question of why have so many groups drawn functions to PGs when they are not present? This was indicative that inflammatory lipid mediators are present, just not of a C20 origin. The whole lipid analysis suggested that *Drosophila* can synthesise C18 inflammatory mediators directly from the C18 precursors linoleic acid and α -linolenic acid. From the literature we identified HODEs as being a possible target because they are directly made from linoleic acid (Vangaveti et al., 2010). It is likely that other C18 lipid mediator variants are present in *Drosophila*, our reasoning for choosing HODEs to investigate was because they are partially characterised and already well documented in an inflammatory disease, atherosclerosis.

In our study, LC-MS analysis of w^{1118} and hop^{Tum} revealed the presence of both 9- and 13-HODE. Interestingly, we observed that supplementation with omega-3 failed to increase the abundance of either HODE above the control level. However, supplementation with omega-6 fatty acid (linoleic acid) markedly increased their abundance in both w^{1118} and hop^{Tum} larvae. This was suggestive that they may play a role in inflammation due to the increase in tumour incidence when hop^{Tum} were raised on an omega-6 diet (Figure 8A). What was surprising was the distinct difference in the level of HODEs in the control w^{1118} compared to the inflamed model hop^{Tum} . Both fly strains had similar levels of 9- and 13-HODE in control and omega-3 supplemented, but there was a marked increase in the levels when supplemented with omega-6 in the w^{1118} strain, more so than the increase seen in the hop^{Tum} strain. We expected to see a larger abundance of 9- and 13-HODE in the inflamed hop^{Tum} flies, but in fact it is the opposite. An explanation for this could be that the w^{1118} flies become overburdened with linoleic acid, and synthesise HODEs to subsequently 'stock pile' them in the phospholipid membrane in an esterified form. The levels of

abundance we have measured do not discriminate between esterified HODEs or free HODEs. It is the free HODEs that would be active, similar to AA; there is very little free AA, which regulates the production of pro-inflammatory mediators. In terms of the *hop^{Tum}* strain, due to the inflamed nature of the fly, it would be disadvantageous to synthesise even more HODEs that would exacerbate the inflammatory phenotype. It is likely that there are inhibitory pathways that are switched on to reduce their production. Conversely, it is possible that there is a rapid turnover of linoleic acid to synthesise HODEs, resulting in an increase of free HODES, that are rapidly oxygenated further to produce 9- and 13-oxooctadecadienoic acids (9- and 13-oxoODEs) (Massey and Nicolaou, 2013). By measuring the levels of oxoODES in *w¹¹¹⁸* and *hop^{Tum}* flies, it would clarify if there were a higher turnover of HODEs in *hop^{Tum}*.

Work in chapter two identified enzymes that potentially produce pro-inflammatory mediators. We observed that the S-enantiomer for both 9- and 13-HODE was present in *w¹¹¹⁸* and *hop^{Tum}* flies at significantly higher levels than the R-enantiomer. This was indicative of enzymatic production because enzymes have a preference for substrates in the S form. For example, COX only abstracts the hydrogen of an S enantiomer of AA (Schneider et al., 2002). When supplemented with omega-6 fatty acid, the abundance of the S form increased suggesting that dietary intake of omega-6 fatty acid can influence the levels of HODEs.

Both the presence and enzymatic production of HODEs posed the question of what their function was in *Drosophila*. As described in section 1.2.4, HODEs have been predominantly documented in atherosclerosis, an inflammatory disease (Waddington et al., 2003; Vangaveti et al., 2010). In particular their involvement in the differentiation of macrophages to lipid-laden foam cells. We too expected to pertain inflammatory effects to HODEs. This in part was due to a similar phenotype seen in

the *hop*^{Tum} mutants that was comparable to that seen in atherosclerosis. The increase in cell size and the noticeable strong lipid staining of *hop*^{Tum} haemocytes was similar to the lipid-laden foam cells seen in atherosclerosis. The increased cell size difference seen in the *hop*^{Tum} strain was not surprising as they have a significant increase in differentiation of lamellocytes that are around 50 micrometres in size. What was surprising, and has never been shown before, is that the lamellocytes are lipid laden posing the possibility that the intake of lipid results in the eventual differentiation of a plasmatocyte into a lamellocyte. Fuelling this theory further was the diminishment of lipid content seen in the *hop*^{Tum} larvae fat body compared to that seen in *w*¹¹¹⁸. It is likely that in this mutant, lipid is liberated from the fat body (analogous of mammalian adipose tissue) and taken up by haemocytes where it can be processed into lipid mediators such as HODEs and cause the eventual differentiation into lamellocytes. The lipid-laden cells aggregate, forming layers that result in a melanised capsule that we show to be largely lipid based similar to an atherosclerotic plaque.

HODEs have been shown to be ligands for PPAR_γ, a nuclear receptor involved in adipogenesis and activation of macrophages (Itoh et al., 2008). We identified *Eip75B* as being a homologue of PPAR_γ. We have shown that deletion of the *Drosophila* homologue *Eip75B* reduces tumour incidence significantly. The Eip75B-LBD reporter assay that was used to conclude HODE actions were elicited though *Eip75B* failed to be conclusive. In the presence of HODEs we expected them to bind to the Eip75B-LBD-GAL4 fusion, and activate the transcription of GFP at the UAS docking site. Surprisingly, GFP reporter activity wasn't achieved in cells cultured with DETA, a nitric oxide (NO) donor. DETA treated cells should have shown reporter activity because NO is known to bind to *Eip75B* and regulate transcription (Marvin et al., 2009). The data is therefore inconclusive and suggests the Eip75B-LBD-GAL4, UAS-

GFP reporter line to be non-functional. For future reference, the possible relationship between *Eip75B* and HODEs could be clarified using a combination of chromatin immune precipitation sequencing (ChIP-seq) and RNA sequencing comparing control and HODE cultured cells.

Although we have not unequivocally determined if HODEs bind to *Eip75B*, we have identified transcriptional targets that pertain to a role in differentiation of haemocytes. In culturing S2 cells with known precursors of immuno-modulatory lipids, omega-3 (α -linolenic acid and omega-6 (linoleic acid) as well as 9- and 13 HODE, we were able to identify potential transcriptional changes. 9-HODE may play a role in upregulating *Iz*. Expression of the transcription factor *Iz* is specific to crystal cell fate. It has also been shown to regulate the binding of Notch, to Notch enhancers, resulting in selected gene activation that locks the cell into a particular differentiation fate (Terriente-Felix et al., 2013). It is possible that 9-HODE specifically functions to direct a cell differentiation fate through *Iz*. However, both 9- and 13-HODE were observed to upregulate *Hml*, a multi-domain protein that is secreted by haemocytes and is involved in clot formation which eventually forms a melanised mass (Lesch et al., 2007). This could be a reason as to why we saw an increase in tumour incidence in *Drosophila* raised on an omega-6 diet, the precursor of 9- and 13-HODE (Figure 8A). It was also shown that both 9- and 13-HODE treated cells showed a marked upregulation of *Itg α PS5*, a marker of lamellocytes (Stofanko et al., 2010). *Itg α PS5* is an alpha integrin, its specific function has not been characterised though it is most likely involved in cell adhesion and have receptor signalling capability. It is uncertain whether HODEs do direct differentiation of lamellocytes due to unchanged expression with other lamellocyte markers *mys*, *Itg α PS4*, *cher* and *Itg β v*, though it is possible that HODEs can elicit aspects of differentiation given the upregulation seen with *Iz*, *Hml* and *He*.

Omega-3 and -6 fatty acid treated cells showed a significantly higher expression in transcript levels of the differentiation markers affected than HODEs. This in part could be due to fatty acid treated cells producing more HODEs, increasing the concentration above that at which we used to treat the HODE treated cells. Therefore eliciting a greater effect on transcript levels of differentiation markers. It is possible that other unknown immuno-inflammatory mediators are synthesised from the omega fatty acids and exerting an effect on gene transcript expression. An example could be dinor isoprostanes that have been documented in plants. These are C18 prostaglandin-like molecules that are formed from α -linolenic acid and have immuno-modulatory effects (Parchmann, 1998).

All cell treatments affected the gene transcript levels of the three housekeeping genes we assayed, *RpL32*, *Gapdh2*, and *α -Tubulin*. Housekeeping genes are used to normalise gene transcript levels because their levels of expression do not change dramatically. With omega fatty acid treatment, notably omega-6 fatty acid treated cells; both *RpL32* and *Gapdh2* transcript levels were considerably increased. We assume that there is an increase in protein translation and metabolic activity because *RpL32* is involved in protein translation and *Gapdh2* is involved in the glycolysis pathway. It is not surprising that there would be an increase in metabolic activity as the cells actively uptake the supplemented fatty acids which in turn requires energy, especially if protein synthesis is increased. By staining omega fatty acid treated cells, we confirmed that an increase seen in gene transcript levels of *He*, resulted in increased protein expression, as the cells stained stronger for He than the control. This suggests that an increase in gene transcript level results in an increase of protein level, adding strength to the increased protein translation that *RpL32* indicates.

To conclude, we have confirmed that AA, EPA and DHA are not present in *Drosophila* and that they are unable to elongate carbon fatty acid chains beyond C18. We validated the absence of PGs through non-detection of PGE₂, PGF_{2α} and PGD₂ using LC-MS. We were able to detect two C18 derivatives of linoleic acid, 9- and 13-HODE. LC-MS chiral analysis established HODEs were enzymatically produced and the level of which could be increased by linoleic acid supplementation. We were unable to conclude HODEs as being a ligand for the *Drosophila* PPAR_γ homologue Eip75b. However, we have identified haemocyte differentiation markers that HODEs upregulate possibly leading to a particular differentiation cell lineage.

3.3 Functional characterisation of the MPO/COX candidates Pxt, Pxn and Irc

We had identified three potential MPO/COX enzymes Pxt, Pxn and Irc that reduced inflammatory tumour incidence when deleted. We speculated that these enzymes have MPO/COX activity and were the source of the inflammatory mediators that exacerbated the inflammatory phenotype. LC-MS analysis had shown the absence of the pro-inflammatory mediator PGs. However, detection of the inflammatory linoleic derivatives HODEs revealed the enzymatic S-enantiomer to be the most prominent indicating an enzymatic route to their synthesis. Given the findings, and the speculation from previous studies that Pxt (one of our candidates) was involved in PG synthesis (Tootle and Spradling, 2008; Groen et al., 2012), it was vital to establish conclusively whether our candidate enzymes had functional COX and MPO activity. We cloned the *Drosophila* MPO/COX candidates, as well as the mammalian COX-1 and COX-2 enzymes and made recombinant fusion proteins to elucidate their *in vitro* activity. We sought to clarify if they can or cannot utilise AA by cyclooxygenase activity and whether they could use hydrogen peroxide as a substrate to determine peroxidase activity. The recombinant protein can also be used to assay for PG and HODE production.

3.3.1 IPTG induction of GST fusion recombinant protein in a bacterial expression system

We initially cloned the *Drosophila* MPO/COX candidates and mammalian COXs into a GST fusion expression vector pGEX-2T, and overexpressed the GST-tagged recombinant protein in *E. coli* using Isopropyl β -D-1-thiogalactopyranoside (IPTG). The pGEX-2T vector utilises the well-characterised lac operon inducible system

(Müller-Hill, 1996). In an un-induced state, binding of a repressor to the operator prevents RNA polymerase elongation and thus the gene of interest is not transcribed (Figure 20A). In the presence of IPTG, it binds to the repressor resulting in a conformational change causing it to disassociate from the operator, allowing RNA polymerase to elongate and transcribe the gene. Overexpression using this system resulted in significant overexpression of our recombinant proteins. Analysis of induced proteins using whole lysates from cells revealed that the proteins of interest were overexpressed abundantly (Figure 20B). However, the majority of this protein was shown to be insoluble, as protein was present in cell pellets when the extracts were centrifuged to pellet insoluble material (Figure 20B). Subsequent modification of the induction and lysis procedures failed to have any significant effect on recovery of soluble protein. The insoluble nature of the protein was likely due to the absence of correct post-translational modification machinery in the bacteria. As we needed functional soluble protein for assaying, we used a mammalian expression system in the hope that the correct posttranslational modifications would be added to over-expressed protein to ensure soluble protein.

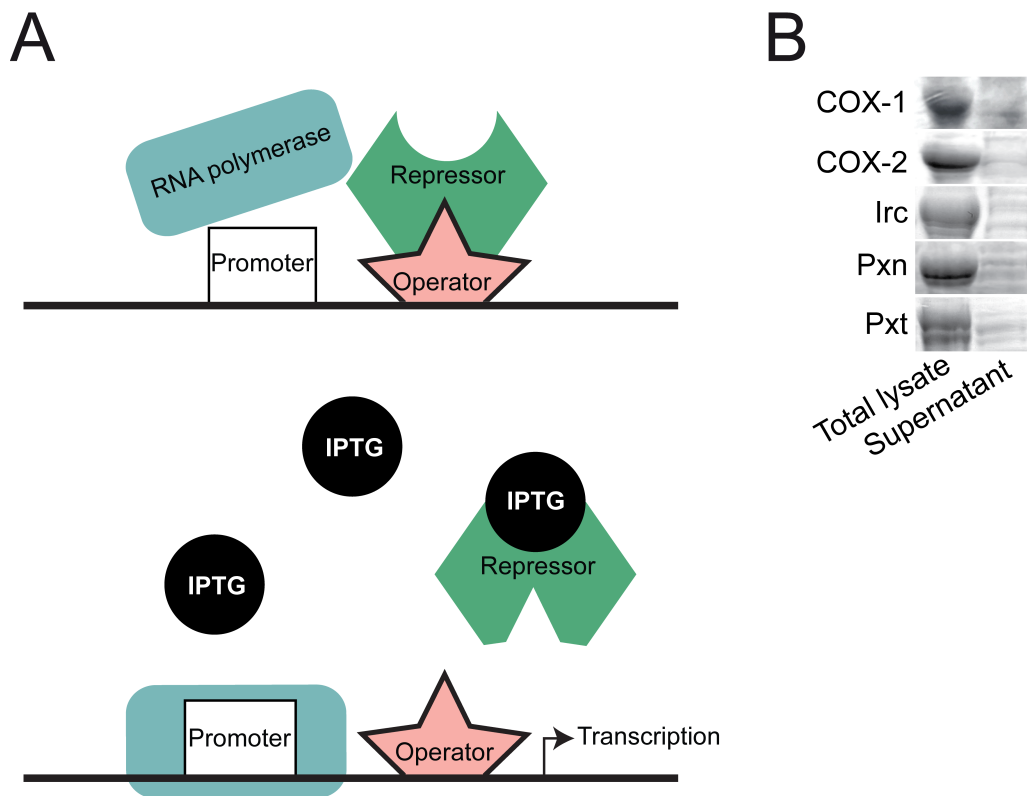


Figure 20. IPTG induction of recombinant protein using the lac operon inducible system

The candidate MPO/COX enzymes Pxn, Pxt, and Irc as well as the mammalian COX-1 and COX-2 controls were overexpressed in *E.coli* using the lac operon inducible system. **(A)** A repressor that binds to the operator prevents RNA polymerase elongation, inhibiting transcription of the target gene. In the presence of IPTG (the inducer), it binds to the repressor relieving inhibitor and allowing RNA polymerase to transcribe. **(B)** Induced cells were analysed for recombinant protein using SDS-PAGE. Both the whole cell lysate and soluble fraction were analysed. The absence of protein in the soluble fraction indicated the protein was insoluble.

3.3.2 Doxycycline induction of FLAG-tagged recombinant protein using the Tet-On 3G inducible expression system

A Tet-On 3G inducible expression system was used to overexpress the recombinant proteins in HeLa cells. The genes of interest were sub-cloned from the pGEX-2T vector and ligated into a pTRE3G expression vector that was engineered to have a 3xFLAG tag that resulted in expression of FLAG-tagged fusion protein. The Tet-on 3G inducible system works by overexpressing a pTRE3G transactivator that is non-functional until the addition of doxycycline (Dox), which binds to the transactivator resulting in a conformational change of the transactivator, which allows it to then bind to tet operator located in the pTRE3G promoter upstream of the gene of interest (Figure 21A). Binding of the transactivator to the pTRE3G promoter initiates transcription of gene. Overexpression of recombinant protein was achieved by induction of Dox in HeLa cells. Subsequent FLAG-affinity purification revealed overexpression of soluble protein for each gene of interest by western blot (Figure 21B). To ensure purification was specific to FLAG, non-transfected HeLA cells were used as a control. The overexpression of soluble recombinant protein allowed for in vitro assays to distinguish the activity of the *Drosophila* candidate MPO/COX homologues. Mammalian COX-1 and COX-2 were cloned and overexpressed in the same way to provide an internal control to compare the activity of *Drosophila* recombinant protein.

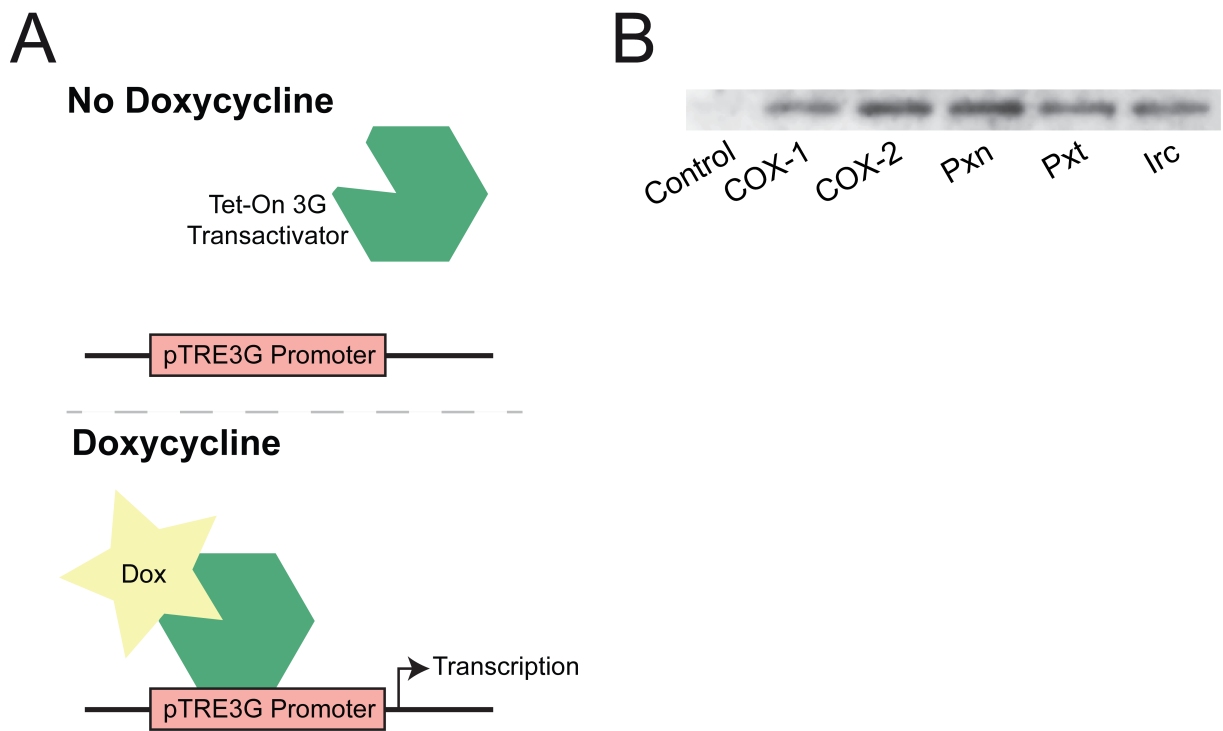


Figure 21. Dox induction of recombinant protein using the Tet-On 3G inducible system

The candidate MPO/COX enzymes Pxn, Pxt, and Irc as well as the mammalian COX-1 and COX-2 controls were overexpressed in HeLa cells using the Tet-On 3G inducible system. **(A)** An overexpressed transactivator is activated when bound to doxycycline (Dox) resulting in a conformational change. The activated transactivator bound to Dox then binds to a tet operator sequence in the pTRE3G promoter sequence and elicits transcription of the downstream gene. **(B)** Purified FLAG-tagged fusion protein was visualised by western using anti-FLAG antibodies. Non-transfected HeLa cells were used as a control.

3.3.3 Determining cyclooxygenase activity of *Drosophila* Pxn, Pxt and Irc *in vitro*

Purified recombinant FLAG-tagged protein enabled us to characterise the *in vitro* activity of the MPO/COX candidates Pxn, Pxt and Irc. We first tested whether these enzymes display *in vitro* COX activity. Although we found that flies lack PGs, we had observed that COX inhibitors suppress inflammatory tumours incidence suggesting that COX-like activity may be present, even if the precursors of PGs are not. We used a fluorometric COX activity assay to determine if the MPO/COX candidates we identified were capable of COX activity. As mentioned previously, the synthesis of AA to PGs is a two-step process. COX utilises AA to produce a first endoperoxide intermediate PGG₂ through cyclooxygenase activity, which is then converted to the PG precursor PGH₂ via peroxidase activity. The fluorometric assay monitors the peroxidase step that converts PGG₂ to PGH₂, by using ADHP (10-acetyl-3,7-dihydroxyphenoxazine), a peroxidase substrate that reduces PGG₂ to PGH₂, and in doing so, ADHP is converted to fluorescent resorufin, which can be measured. As shown in Figure 22A, we observed significant COX activity from the three *Drosophila* MPO/COX candidates. The activity was comparable with that seen with the COX-1 and COX-2 internal standards, and the COX-1 standard provided with the kit (Figure 22A). We showed that COX activity required AA addition as only basal COX activity was detected without the addition of AA. This suggested that the *Drosophila* candidates were able to utilise AA and possess cyclooxygenase activity.

To further clarify the presence of peroxidase activity and the candidate MPO/COX enzyme's ability to utilise hydroperoxides, we assayed peroxidase activity using hydrogen peroxide. The fluorometric assay used above was modified in that hydrogen peroxide was added as a substrate rather than AA. This directly assays peroxidase activity. We observed significant peroxidase activity with the addition of

hydrogen peroxide (H_2O_2) (Figure 22B). The activity seen was similar across all the enzymes assayed. It was shown that addition of an MPO inhibitor markedly inhibited all peroxidase activity. The results suggested that Pxn, Pxt and Irc could utilise hydrogen peroxide as a substrate and that they have peroxidase activity, which can be inhibited by an MPO inhibitor.

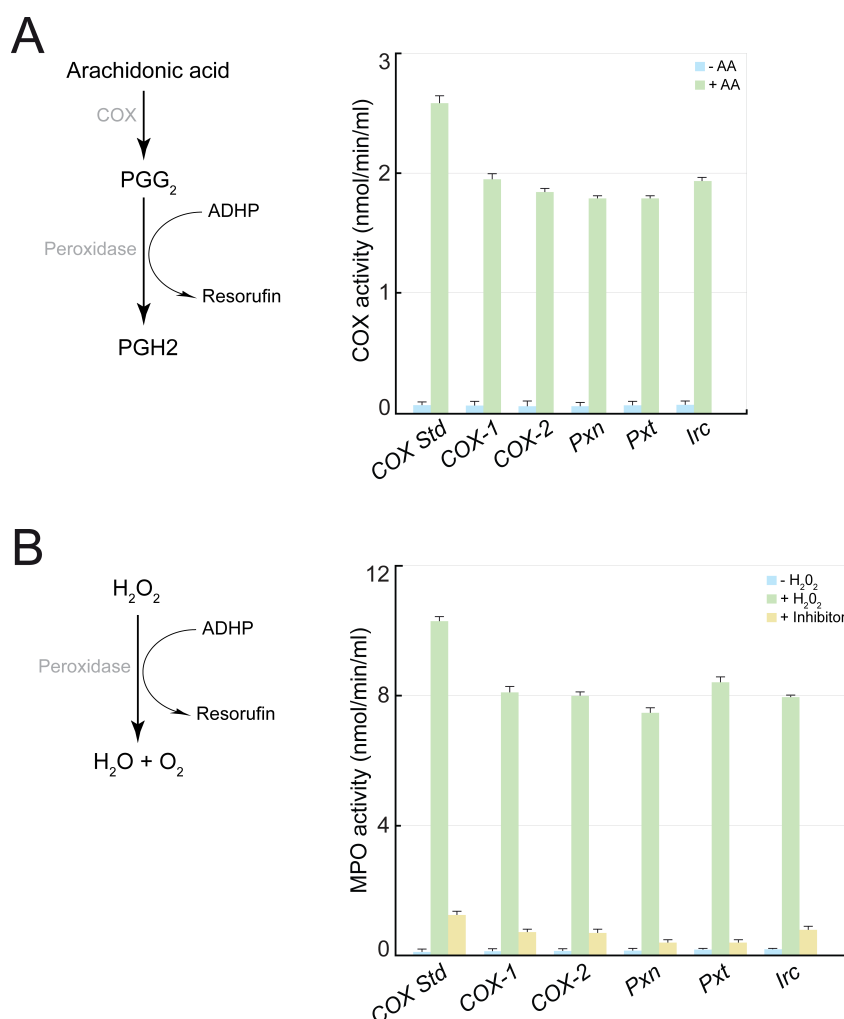


Figure 22. Fluorometric assay determining COX and MPO activity

(A) A fluorometric assay was used to measure COX activity of the three *Drosophila* MPO/COX candidates and mammalian COX-1 and COX-2 enzymes with the addition of AA. The assay monitors the peroxidase step that converts PGG_2 to PGH_2 by using ADHP (10-acetyl-3,7-dihydroxyphenoxazine), a peroxidase substrate that is used to reduce PGG_2 to PGH_2 and in doing so, is converted to the highly fluorescent resorufin, which can be measured. **(B)** Peroxidase activity and the utilisation of hydroperoxides, using hydrogen peroxide was measured. In this assay, hydrogen peroxide is reduced by ADHP converting it to fluorescent resorufin and converting hydrogen peroxide to two molecules of water and an oxygen molecule. MPO Inhibitor-1 was used to measure inhibition of peroxidase activity. $n=5$

3.3.4 Discussion

We have shown that the MPO/COX candidates Pxn, Pxt and Irc that were highlighted for their amino acid sequence similarity to mammalian COX, can utilise AA to produce the PG precursor PGH₂, indicating that they possess COX activity *in vitro*. The detection of COX activity from the *Drosophila* candidate enzymes suggests that they are able to emulate the actions of COX-1 and -2. It is an indication that the candidates must be able to radicalise a residue to initiate the COX reaction. Consistent with this, the Tyr385 residue that is radicalised to abstract the C13 hydrogen from AA is conserved in Pxn, Pxt and Irc (Rogge et al., 2006; Wu et al., 2011). We further demonstrated that the *Drosophila* candidates could utilise hydrogen peroxide as a substrate by their peroxidase activity, the second step to forming the PGH₂ precursor to PGs. MPO activity of the candidates was successfully inhibited by MPO Inhibitor-1, suggesting the reduction in tumour incidence seen with MPO Inhibitor 1 from the tumour assay potentially was due to inhibition of Pxn, Pxt and Irc in producing pro-inflammatory mediators.

Interestingly, although Pxn, Pxt and Irc can utilise AA *in vitro*, our LC-MS data showed that AA is not present *in vivo*. Although these enzymes have COX activity, the absence of precursor results in absence of PGs. It is likely that the *Drosophila* candidate MPO/COX enzymes can oxygenate a number of fatty acid substrates. Although mammalian COX-1 and -2 preferentially oxygenate AA, they also can oxygenate a number of substrates, though less efficiently (Laneuville et al., 1995). COX-2 is well documented as the most promiscuous in its oxygenation of substrates due to differences in the residues found in the substrate-binding pocket compared to COX-1 (Vecchio et al., 2012). In particular COX-1 and COX-2 can oxygenate linoleic acid to generate 9- and 13-HODE. We would suggest that Pxn, Pxt, and Irc are also

substrate promiscuous, and although AA is not found *in vivo*, this would explain why they could utilise AA *in vitro*.

The recombinant protein of the *Drosophila* MPO/COX candidates allows for an additional *in vitro* experiments to be conducted. As of yet, we have only partially characterised the enzymes. We suspect that incubation of the candidate enzymes with linoleic acid would result in production of HODEs. Given that HODEs are known to be synthesised from COX-1 and COX-2 (Vecchio et al., 2012), this would provide a route for enzymatic HODE production in *Drosophila*. Considering inhibition of COX has been shown to prevent the production of HODEs (Engels et al., 1991), this could explain why a reduction in tumour incidence was seen with both non-specific and specific COX-1 and -2 inhibitors in our tumour assay.

In conclusion, we have shown that the *Drosophila* MPO/COX candidates have cyclooxygenase and peroxidase activity. *In vitro* they are able to utilise AA to produce the precursor to PGs, PGH₂. Due to absence of AA *in vivo*, we speculate that like mammalian COX enzymes, the *Drosophila* candidates can oxygenate C18 substrates. These data suggest that the absence of PGs *in vivo* is not due to absence of COX activity, but rather, the absence of the C20 precursor AA. We speculate the *Drosophila* candidates Pxn, Pxt and Irc to be the source of HODEs.

3.4 In vivo characterisation of Pxn, Pxt, and Irc

We have shown that inflammatory tumour number can be reduced with the use of MPO and COX inhibitors. MPO/COX-like *Drosophila* candidate deletions have also reduced inflammatory tumour incidence. These candidates, Pxn, Pxt and Irc have been observed to have MPO and COX activity by in vitro characterisation, suggesting a role in producing lipid immuno-modulators. Lipid mediators are well known to be involved in numerous homeostatic functions within animals, and are especially important during inflammation to aid combat infection and mediate tissue repair (Pentland and Needleman, 1986; Goldmann et al., 2010). Our data suggests that the *Drosophila* MPO/COX candidates play a role in producing lipid mediators involved in homeostatic functions as well as inflammation. We used deletions of the *Drosophila* candidates Pxn, Pxt and Irc, to determine the affect on the survival of the animals in response to a range of stimuli. Longevity of flies raised in standard conditions, after being physically stressed (shaken), sterile wounded, and after infection with fungi, Gram-positive and a Gram-negative bacteria. We anticipated, that the lack of lipid mediator production due to deletion of MPO/COX candidates would have a significant impact on survival.

To reduce levels of Pxt and Irc, we used transposon element insertions for *Pxt* and *Irc* as was used in the tumour assay. Due to the large size of the chromosomal deletion used for *Pxn* in the tumour assay, we used RNAi driven by a blood specific GAL4 line, *Hml-GAL4* to ablate *Pxn*, which is predominantly expressed in blood cells. RT-PCR verified knockdown of *Pxn* by over 10 fold. The GAL4 driver *Hml-GAL4* alone was used as a control for *Pxn* knockdown experiments, and *w¹¹¹⁸* was used for *Pxt* and *Irc*.

3.4.1 The effect of *Pxn*, *Pxt*, and *Irc* knockdown on *Drosophila* ageing and survival in response to sterile wounding

We first assayed longevity of MPO/COX knockdown strains in response to three different sterile stimuli. To assay longevity, *Drosophila* strains were aged for 3-5 days and incubated at 25°C throughout the assay, and survival recorded every 24 hours. We first measured the effects of the MPO/COX candidate knockdowns on longevity while the flies were raised in standard control conditions. We observed a 33% reduction in longevity with the *Pxt* knockdown (Figure 23). Whereas reduction of *Irc* and *Pxn* were shown to significantly increase the longevity of the *Drosophila* by 28% and an impressive 48% with *Pxn* knockdown compared to the controls. In contrast, when flies were shaken in a sterile petri dish for 10 seconds and then raised in control conditions, both *Irc* and *Pxn* deficient flies were strongly affected (Figure 23). *Pxn* showed a 13.8% reduction in survival, and *Irc* was the most effected having a 37% reduction in longevity compared to controls. *Pxt* on the other hand although showed a significant reduction in survival compared to the *w¹¹¹⁸* control, its survival was comparable to that seen with *Pxt* knockdown in the ageing assay. When fly strains were wounded between the A4-A5 dorsal abdominal tergites with sterile 1xPBS, we observed all three deletions to have reduced survival compared to the controls (Figure 23). The sterile longevity assays suggest removal of *Pxn* and *Irc* can be beneficial in control conditions, but are required when the fly is subjected to stress damage and wounding. In contrast flies with reduced *Pxt* levels show equivalent reduction in survival in all three assays, indicating a potential important homeostatic role that is fundamental to the fly's survival.

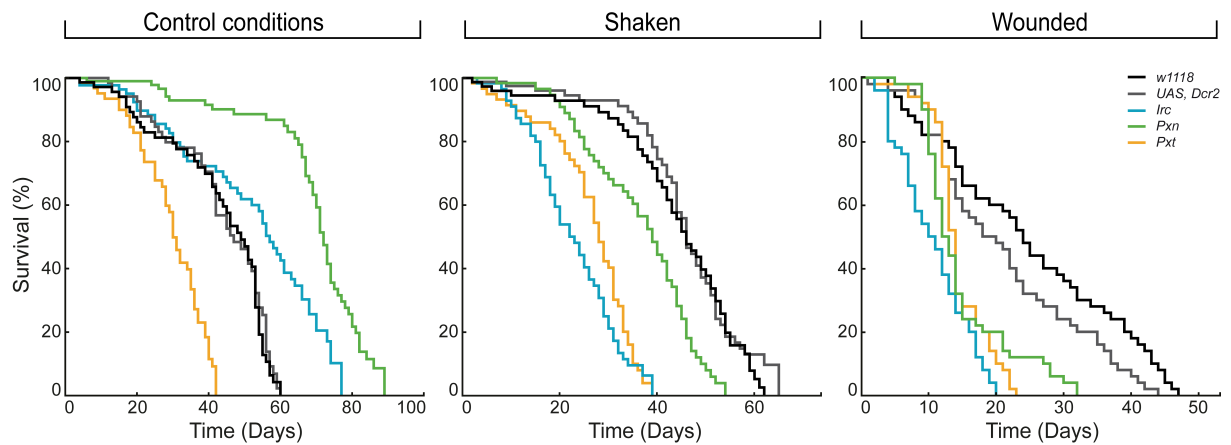


Figure 23. The effect Pxn, Pxt, and Irc knockdown on *Drosophila* ageing and survival in response to sterile wounding

Kaplan Meier graphs showing the survival of male *Drosophila* deficient in the MPO/COX candidates were assayed for longevity. The transposon element insertions *Pxt*^{#05258} and *Irc*^{MB11278} were used to delete *Pxt* and *Irc*. For *Pxn*, the *UAS-Pxn* RNAi line driven by *UAS-Dcr2*, *Hml-GAL4* was used to knockdown *Pxn*. *w*¹¹¹⁸ and *UAS-Dcr2*, *Hml-GAL4* (alone) were used as controls. The mutants were assayed under normal control conditions to determine effects on longevity. The shaken assay was conducted by shaking flies for 10 seconds in a sterile petri dish and then raised in normal control conditions. The wounding assay was conducted using a fine sterilised platinum needle, which was used to wound flies between the A4-A5 dorsal abdominal tergites with sterile 1xPBS. Wounded flies were then raised in standard control conditions. Flies were assayed for survival every 24 hours. 5 groups of 50 flies were assayed for each strain and the mean survival calculated per point. Kaplan Meier graphs were generated using SPSS.

3.4.2 The effect of *Pxn*, *Pxt*, and *Irc* knockdown on survival in response to infection

It was anticipated that *Pxn*, *Pxt* and *Irc* would be fundamental in the response to an infection as the synthesis of immuno-modulator lipid mediators is important to orchestrate the clearance of pathogen and initiating tissue repair. Thus, knockdown of these genes would be expected to have a significant affect on the survival of the animal in response to infection. To determine whether *Pxn*, *Pxt* and *Irc* are required during an infection, we monitored survival of knockdown strains in response to *Drosophila* pathogens. We assayed three pathogen groups, fungi, Gram-negative and Gram-positive bacteria. *Beauveria bassiana* is an entomopathogenic fungal pathogen of *Drosophila* that infects *Drosophila* when its spores attach to the cuticle of the fly, and uses proteases to penetrate the fly (Bidochka and Khachatourians, 1994; Joshi et al., 1995). We grew *B.bassiana* on potato and carrot agar plates for 3 days at which point peak sporulation occurs. The *Drosophila* strains were shaken gently over the cultured plate for 10 seconds to infect them with spores, and then raised under normal control conditions. As a control, flies were shaken over sterile potato and carrot agar plates.

We observed that infection of flies with *B.bassiana* strongly reduced survival of the *Pxt* mutant by 73% compared to infected *w¹¹¹⁸* (Figure 24A). *Irc* also showed a reduction in survival of 45%. Surprisingly, the *Pxn* mutants had an increased survival upon infection of 22% compared to the infected control line. Although *Pxn* mutants initially died faster than the uninfected *Pxn* mutants, by day 42 both the infected and uninfected *Pxn* mutants displayed identical survival. RT-PCR analysis of *w¹¹¹⁸* infected, and non-infected flies revealed that *B.bassiana* infection does not affect *Pxn* and *Pxt* transcript levels (Figure 24B). However, *Irc* was slightly upregulated in

infected flies suggesting that induction of *Irc* expression may play a role in the ability of the fly to overcome an infection.

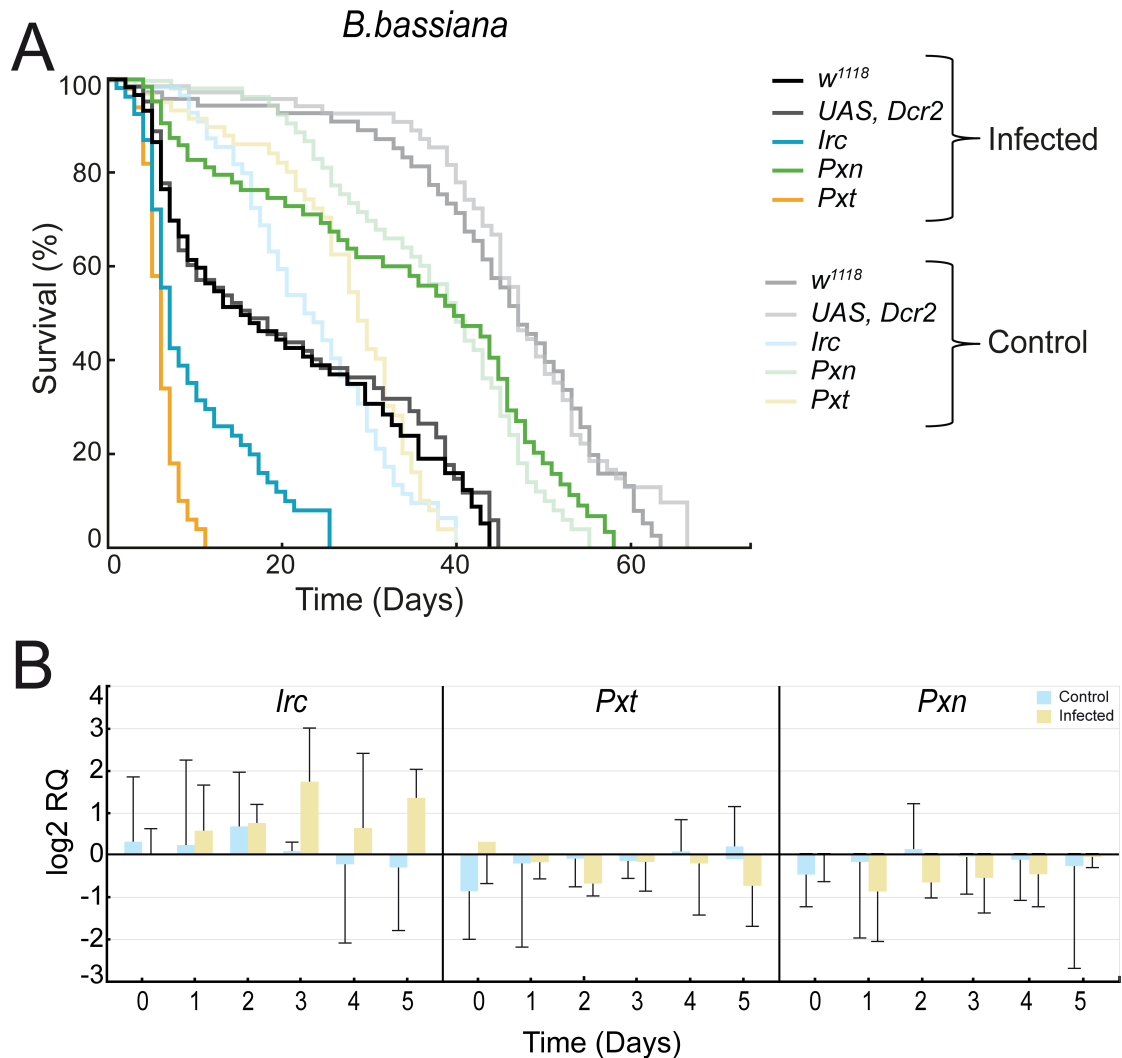


Figure 24. The effect of *B.bassiana* infection on *Pxn*, *Pxt*, and *Irc* mutant survival

Kaplan Meier graphs showing the survival of male *Drosophila* deficient in the MPO/COX candidates were assayed for longevity after infection with *B.bassiana*. The transposon element insertions *Pxt*¹⁰⁵²⁵⁸ and *Irc*^{MB11278} were used to delete *Pxt* and *Irc*. For *Pxn*, the *UAS-Pxn* RNAi line driven by *UAS-Dcr2*, *Hml-GAL4* was used to knockdown *Pxn*. w^{1118} and *UAS-Dcr2*, *Hml-GAL4* (alone) was used as controls to compare survival to. **(A)** The flies were shaken on a 3-day-old *B.bassiana* plate for 10 seconds and then raised under normal control conditions. Control flies were shaken on a sterile potato and agar plate. Longevity was assayed every 24 hours. **(B)** Five w^{1118} control and five *B.bassiana* infected flies were collected for RNA extraction after 0, 1, 2, 3, 4, and 5 days, cDNA synthesised and *Pxn*, *Pxt* and *Irc* transcript levels measured against *RpL32*. 5 groups of 50 flies were assayed for each strain and the mean survival calculated per point. Kaplan Meier graphs were generated using SPSS.

Pseudomonas entomophila, an entomopathogenic Gram-negative bacterium that is highly pathogenic to *Drosophila* (Vodovar et al., 2005), was used to elucidate whether deletions in *Pxn*, *Pxt* and *Irc* affected the survival of the fly in response to a Gram-negative pathogen. It is not entirely understood how *P.entomophila* infects and causes the eventual death of *Drosophila*. It is thought that secretion of insecticidal toxins such as PSEEN2485 and PSEEN2697 play a significant role in the process (Vodovar et al., 2006). We cultured *P.entomophila* on agar containing 10% non-fat dried milk and pathogen colonies were selected by its protease activity. A liquid culture was subsequently made, and a working stock of OD_{0.1} was used to wound the different *Drosophila* strains. As a control, flies were also wounded with 1xPBS and both infected and non-infected flies were raised in normal control conditions and the survival assayed every 6 hours.

We observed, as expected that all *Drosophila* strains were affected by infection with *P.entomophila* compared to non-infected. Curiously, we observed that *Pxn*, *Pxt* and *Irc* deletions, although sensitive to pathogen, survived significantly longer than the wild type *w¹¹¹⁸* and *UAS, Dcr2* flies (Figure 25A). Both *Pxt* and *Irc* mutant flies survived 33 hours longer than *w¹¹¹⁸* infected flies, whereas *Pxn* mutant flies survived even longer, having an increase in longevity by 156 hours. RT-PCR analysis confirmed that *Pxn*, *Pxt* and *Irc* were induced during infection of *w¹¹¹⁸* (control) flies (compare infected to non-infected flies in Figure 25B). *Irc* and *Pxt* transcript levels were induced 9-fold while *Pxn* transcript levels were increased 4-fold. The data suggests that production of *Pxt*, *Irc* and especially *Pxn* may be detrimental to the fly's survival during an infection of *P.entomophila*, as knockdowns increase survival compared to control flies.

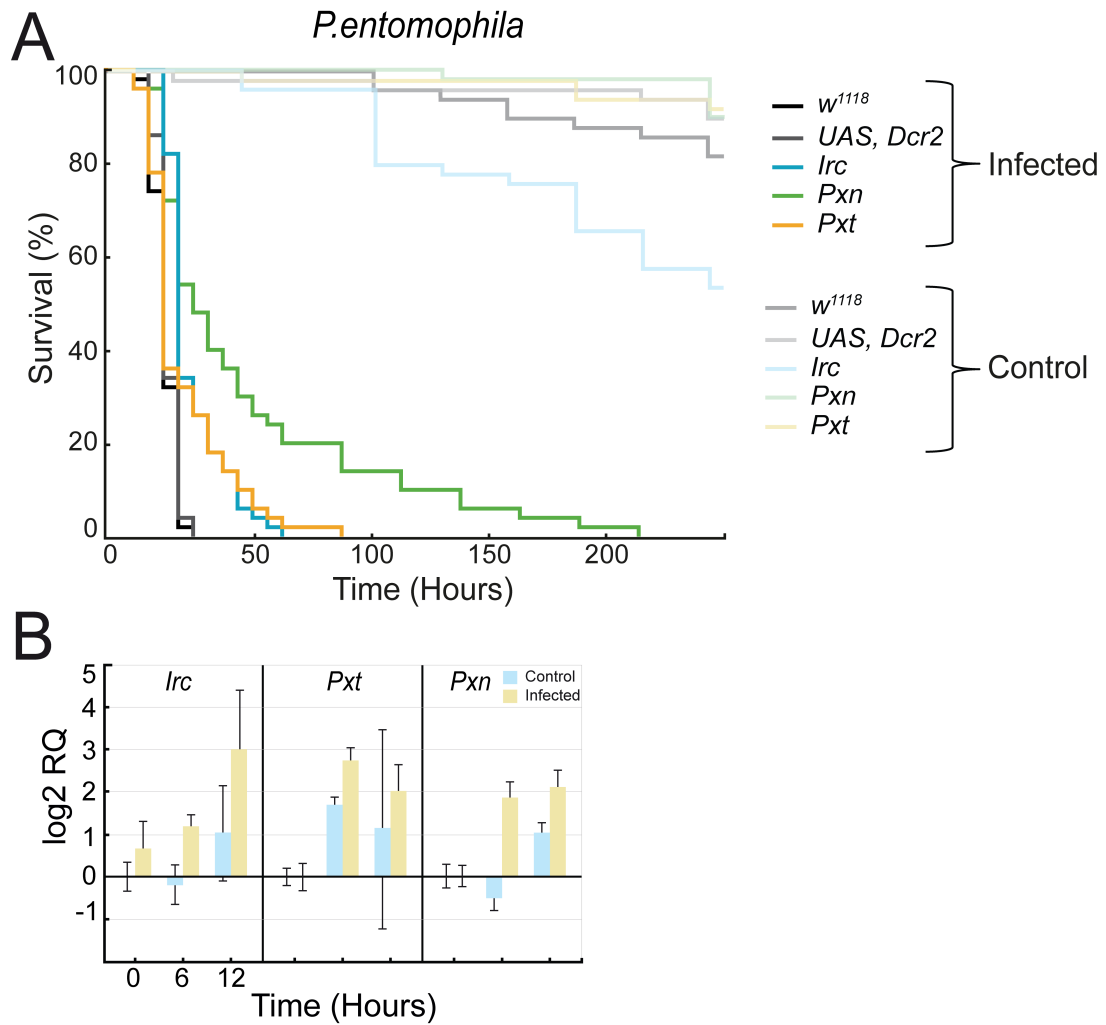


Figure 25. The effect of *P.entomophila* infection on *Pxn*, *Pxt*, and *Irc* mutant survival

Kaplan Meier graphs showing the survival of male *Drosophila* deficient in the MPO/COX candidates were assayed for longevity after infection with the Gram-negative bacterium, *P.entomophila*. The transposon element insertions *Pxt*^{f05258} and *Irc*^{MB11278} were used to delete *Pxt* and *Irc*. For *Pxn*, the *UAS-Pxn* RNAi line driven by *UAS-Dcr2*, *Hml-GAL4* was used to knockdown *Pxn*. w^{1118} and *UAS-Dcr2*, *Hml-GAL4* (alone) was used as controls to compare survival to. **(A)** The flies were wounded with *P.entomophila* of an OD_{0.1} using a sterile platinum needle and then raised under normal control conditions. Control flies were wounded in the same way, but with sterile 1xPBS. Longevity was assayed every 6 hours. **(B)** Five w^{1118} control, and five *P.entomophila* infected flies were collected for RNA extraction after 0, 6 and 12 hours, cDNA synthesised, and *Pxn*, *Pxt* and *Irc* transcript levels measured against *RpL32*. 5 groups of 50 flies were assayed for each strain and the mean survival calculated per point. Kaplan Meier graphs were generated using SPSS.

To elucidate if the *Drosophila* MPO/COX candidates played a role during a Gram-positive bacterial infection; we observed the effects on the survival of these mutants in response to *Enterococcus faecalis*. *E.faecalis* is an entomopathogenic bacterium and is best characterised for its infection of the bowels, in which *Drosophila* have been used extensively as a model for mammalian gut infection (Apidianakis and Rahme, 2011). *E.faecalis* was cultured, and a working stock of OD₁₀ was used to wound the different *Drosophila* strains. Non-infected flies were wounded in the same way but with sterile 1xPBS. Both infected and non-infected flies were assayed for survival every 24 hours.

In infecting with *E.faecalis*, we observed a similar trend seen with *P.entomophila* infection. All three MPO/COX candidate mutants showed increased survival compared to wild type. *Pxn*, *Pxt* and *Irc* each survived an extra four days compared to wild type (Figure 26A). Wild type survival plummets during the first three days of infection, whereas the reduction in survival of MPO/COX candidate mutant's is more gradual. RT-PCR analysis of *E.faecalis* w¹¹¹⁸ infected and non-infected flies revealed that *Irc* expression was induced by 9-fold in control wounding, and 16-fold after infection (Figure 26B). However, *Pxn* induction was 5-fold higher in un-infected flies than seen with *E.faecalis* infected *Drosophila*. This may not be surprising, because *Pxn* itself is secreted from blood cells and is involved in tissue genesis (Bhave et al., 2012). Conversely, *Pxt* induction was largely variable for both infected and sterile wounded wild type flies. The results suggest *Irc* and *Pxn* may play a role in overcoming Gram-positive bacterial infections. However, it is difficult to disentangle whether the induction is a response to the infection, or simply a response to the wounding.

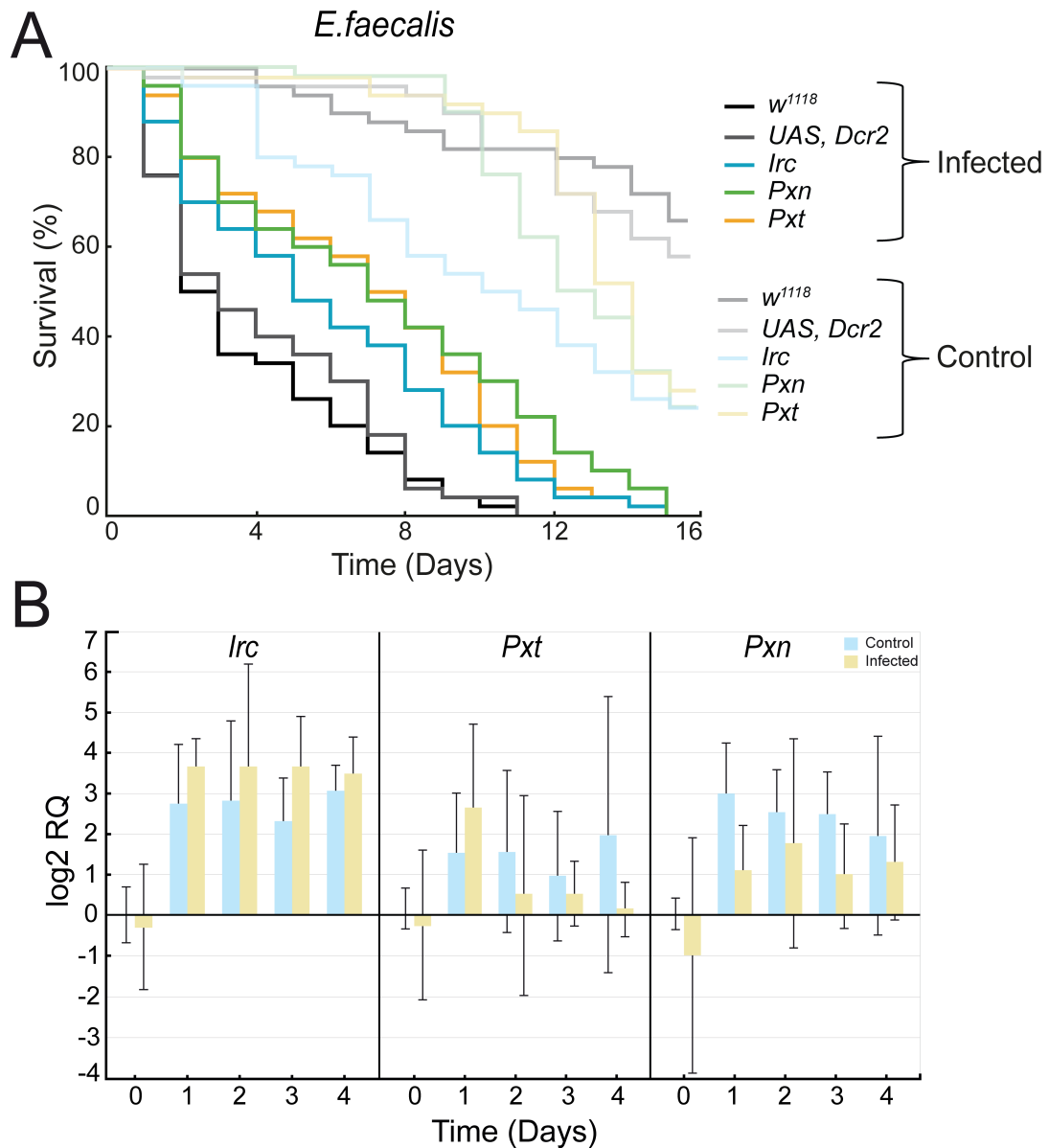


Figure 26. The effect of *E. faecalis* infection on *Pxn*, *Pxt* and *Irc* mutant survival

Kaplan Meier graphs showing the survival of male *Drosophila* deficient in the MPO/COX candidates were assayed for longevity after infected with the Gram-positive bacterium, *E. faecalis*. The transposon element insertions *Pxt*^{f05258} and *Irc*^{MB11278} were used to delete *Pxt* and *Irc*. For *Pxn*, the *UAS-Pxn* RNAi line driven by *UAS-Dcr2*, *Hml-GAL4* was used to knockdown *Pxn*. w^{1118} and *UAS-Dcr2*, *Hml-GAL4* (alone) was used as controls to compare survival to. **(A)** The flies were wounded with *E. faecalis* of an OD₁₀ using a sterile platinum needle and then raised in normal control conditions. Control flies were wounded in the same way, but with sterile 1xPBS. Longevity was assayed every 24 hours. **(B)** Five w^{1118} control, and five *E. faecalis* infected flies were collected for RNA extraction after 0, 1, 2, 3, and 4 days, cDNA synthesised, and *Pxn*, *Pxt* and *Irc* transcript levels measured against *RpL32*. 5 groups of 50 flies were assayed for each strain and the mean survival calculated per point. Kaplan Meier graphs were generated using SPSS

3.4.3 Discussion

Lipid inflammatory mediators are an important part of immunity especially in response to an infection or tissue damage. We anticipate that deletion of *Drosophila* MPO/COX homologues would have severe effects on the survival of the flies. This in part would be due to their inability to produce lipid mediators that are required for actions such as activating macrophages and chemotaxis of blood cells. We observed variable effects between Pxn, Pxt and Irc mutants using different assays.

Pxt mutant flies showed a reduction in survival in the absence of infection. This suggested that Pxt has a homeostatic function that is fundamental to the survival of the fly. In contrast, *Pxt* knockdown increased the fly's survival during both Gram-negative and Gram-positive bacterial infection compared to infected control, but was severely affected during fungal infection. An explanation for the weak survival seen in the Pxt mutant could be due to actin defects. It has been shown that Fascin-1, an actin bundling protein is a downstream target of Pxt (Groen et al., 2012). Fascin-1 is regulated by downstream pathways of PGs (Adams and Schwartz, 2000; Yamakita, 1997), which Pxt is believed to synthesise, but we have shown PGs to be absent in *Drosophila*, so it is possible that Pxt mediates Fascin-1 through a C18 lipid mediator such as HODEs. Pxt mutants share identical phenotypes to Fascin-1 mutants, whereby actin remodelling is largely affected, showing a reduction in actin bundling. It is possible that the effects seen with actin remodelling in Pxt mutants are the reason for its poor survival (Groen et al., 2012). Fascin-1 is involved in the formation of lamellipodia that is required for cell motility (Adams, 1997). It is possible that in the Pxt mutants, given the lack of actin bundling, there could be reduced haemocyte motility, rendering them inert and unable to help clear infection and repair tissue damage. Actin-remodelling dysfunction could also explain why there is a severe reduction in survival with *B.bassiana* infection. Due to reduction in actin bundling, it is

possible that there is a defect in the basement membrane, allowing the hyphae of the fungus to penetrate more easily.

Reduction in Pxn levels significantly increased longevity of the fly in standard control conditions. Survival analysis revealed Pxn could be involved in tissue repair responses because survival of the Pxn mutants was greatly reduced during shaken and wounded assays. During the shaken and wounding assay, extensive tissue damage is inevitable; the inability for the fly to repair this could lead to its reduced survival. Pxn mutants have been observed to have disordered collagen IV networks, an important component of the basement membrane, that is strengthened by sulfilimine bonds (Vanacore et al., 2009). Pxn has been shown to form sulfilimine bonds through the generation of hypohalous acids, highlighting a role in tissue biogenesis (Bhave et al., 2012). It is therefore possible that while *Drosophila* may survive longer without Pxn, if *Drosophila* encounters tissue damage, their ability to repair the damage is compromised resulting in a reduced survival. Pxn was induced during control wounding, which would also add to the theory that it is involved in tissue repair. Pxn may play an active role in the immune response towards both Gram-negative and Gram-positive bacteria due to its induced expression during infection of these pathogens. However, it is unclear why Pxn deletion results in an increase in survival in response to fungal infection, especially as it was not induced during this infection.

As with Pxn, we expect *Irc* mutants are involved in tissue damage responses as we observed an increased longevity during standard control conditions, but was reduced when shaken and sterile wounded. It suggested that the animals were unable to heal and therefore lead to an increased mortality. We had shown that all three MPO/COX candidates have MPO activity. MPO can convert hydrogen peroxide into several different toxic species, the major oxidant being hypochlorous acid (HOCL) (Spickett

et al., 2000). MPO is upregulated during infection to produce the toxic products to aid in pathogen killing (Christensen & Rothstein, 1985). It is likely that *Pxn*, *Pxt* and *Irc* have a similar role as we observed induction of these in response to both bacterial infections. We suggest that during these infections, wild type flies die faster because of a rapid acute surge in the production of highly reactive species. Although the reactive compounds are produced to combat infection, they are extremely damaging to the hosts tissues (Bergamini et al., 2004).

During infection, hydrogen peroxide is actively released by blood cells as a means to kill the pathogen (Babior, 1984). As part of a feedback loop, PG production is induced by hydrogen peroxide during infection (Murthy et al., 1990). An increase in survival of *Pxn*, *Pxt* and *Irc* during bacterial infections could therefore be due to a lack of lipid-mediator production. It is possible that in the wild type, lipid mediators are induced by either hydrogen peroxide or by other means, to create a potent immune response that overwhelms the fly, resulting in reduced longevity.

To conclude, our results have shown an interesting insight into the role *Pxn*, *Pxt* and *Irc* play during *Drosophila* longevity and infection. We believe that they are involved in immune responses that are potentially mediated through the production of lipid mediators such as HODEs. For instance, although it is claimed actin dysregulation is mediated through PG signalling in *Drosophila*, it is possible that it is actually HODEs mediating actin remodelling. Further clarification is needed to establish whether lipid mediators such as HODEs are involved in immune responses against pathogens.

4. Discussion

Inflammatory disease is on the rise due to a combination of lifestyle, environment and genetic predisposition. Inflammatory diseases have extensive affects on health and economic costs. For example, cardiovascular disease (CVD) alone, is responsible for 40% of all deaths in the EU and costs the EU economy approximately €196 million per year (European Society of Cardiology). There is a requirement for new therapeutic targets to be developed and *in vivo* models that can be used to dissect underlying inflammatory mechanisms. Mouse models are currently the go-to for *in vivo* modelling of inflammatory diseases such as atherosclerosis and rheumatoid arthritis (Daugherty, 2002; Asquith et al., 2009). We believe *Drosophila* could be a useful genetically tractable, cheap alternative tool in aiding inflammatory research.

Consistent with other inflammatory models that rely on a mutant background to initiate an inflammatory insult that can be used to disentangle mechanisms that regulate the phenotype, such as the apolipoprotein deficient mouse used to study atherosclerosis (Meir and Leitersdorf, 2004), we too have used a mutant *Drosophila* model that provides an extremely useful model for inflammation. We had used the *hop*^{Tum} mutant in a similar way to initiate an inflammatory response, by overstimulating the JAK/STAT pathway resulting in the formation of an inflammatory melanotic tumour (an aggregation of blood cells) (Harrison et al., 1995). This phenotype aided us in identifying downstream lipid mediators and the presence of MPO and COX activity.

Prostaglandins have been at the forefront of the inflammatory field due to their involvement in inflammation (Ricciotti and FitzGerald, 2011). This in part was fuelled by the discovery of aspirin's (a non-steroidal anti-inflammatory drug) actions, which

alleviates inflammation through inhibiting COX and preventing production of prostaglandins (Vane, 1971). COX soon became a popular therapeutic target for inflammation. However, prostaglandins were soon discovered to have a wide range of homeostatic functions that are fundamental to an animal's health. Inhibition of COX, although reduces inflammation, it impinges on the housekeeping functions of prostaglandins resulting in adverse affects particularly seen with COX-2 inhibition (Wong et al., 2005).

The existence of prostaglandins in other systems has been expected due to the numerous functions of prostaglandins. There has been much speculation of the presence of prostaglandins and arachidonic acid (AA) in invertebrates, including *Drosophila*. We have clarified that *Drosophila* lack AA, the important rate determining fatty acid of eicosanoid production (Norman, 2000). We have shown that *Drosophila* lack the ability to elongate beyond C18 fatty acids, but it is possible that *Drosophila* need to acquire AA through their diet as do cats (Bauer, 2007). The fact that AA is detected in *Drosophila* raised on AA supplemented media, and that they are able to convert C22 fatty acids into AA gives light to a possible dietary requirement (Shen et al., 2010). We believe this is not true, because our LC-MS data validated the distinct lack of PGE₂, PGD₂ and PGF_{2a}, the derivatives of AA. The lack of PGs in *Drosophila* is contrary to previous studies that have suggested PGs have physiological roles in flies (Groen et al., 2012; Tootle and Spradling, 2008; Carton et al., 2002).

It is likely that the physiological roles PGs have been attributed to in *Drosophila* is actually through C18 fatty acid derivatives. We have shown that flies have hydroxy-octadecadienoic acids (HODEs), 9- and 13-HODE in which the enzymatic S form is the most abundant. HODEs have been documented to have immuno-modulatory effects and have been most studied in atherosclerosis (Vangaveti et al., 2010;

Waddington et al., 2003; Henricks et al., 1991). They are produced by COX, and inhibition of COX reduces levels of HODEs (Engels et al. 1991). We find that treatment of *hop*^{Tum} flies with COX inhibitors reduces the inflammatory phenotype. Although HODEs are the most abundant oxidation product found in plaques (Waddington et al., 2003), their role in atherosclerosis is not well characterised. *Drosophila* provides an excellent model system to study the downstream targets of HODEs and to dissect their actions and molecular mechanisms. What now makes *Drosophila* an ever better model, is that they lack C20 lipid mediators that often overshadow the effects of C18 lipid mediators in mammals.

5. Conclusion

In this work, we have used the *hop*^{Tum} *Drosophila* mutant as a model to elucidate downstream lipid mediator signals that are involved in inflammation. The *hop*^{Tum} mutant initiates an initial inflammatory insult through constitutive activation of the JAK/STAT pathway that results in an inflammatory melanotic tumour (aggregation of blood cells). This phenotype aided in assaying for enhancers and suppressors of the inflammatory phenotype.

We have shown that dietary supplementation of polyunsaturated fatty acids, linoleic acid and α -linolenic acid affects tumour incidence, whereby α -linolenic acid was shown to suppress the effects of linoleic acid. In contrast, supplementation with saturated fat and cholesterol had little effect on the tumour incidence. The use of both non-selective and specific COX-1 and -2 inhibitors as well as MPO-1 inhibitor significantly reduced tumour number suggesting the presence of MPO or COX activity. We identified three *Drosophila* proteins that were homologous to COX-1, COX-2 and MPO, namely *Pxn*, *Irc* and *Pxt*. Deletions of these genes showed substantial reduction in the incidence of inflammatory tumour number. We have shown that the liberation of free fatty acids i.e. the precursors to lipid inflammatory mediators is through a combination of PLC and sPLA2 hydrolysis.

We used LC-MS to identify that *Drosophila* lack AA, consistent with other studies, showing that *Drosophila* are unable to elongate fatty acid chains beyond C18. Contrary to speculation in the literature, we have shown that *Drosophila* also lacks the prostaglandins PGE₂, PGD₂ and PGF₂. However, we identified 9- and 13-HODE, C18 oxygenated derivatives of linoleic acid, and demonstrated that the abundance of these could be increased with dietary linoleic supplementation. We showed that

HODEs were enzymatically produced given that the chiral S-enantiomer of 9- and 13-HODE was most dominant. 9-HODE was also shown to be the most abundant in *Drosophila* compared to 13-HODE. We identified a *Drosophila* PPAR γ homologue, (*Eip75b*) as a potential receptor for HODEs. We showed that *Eip75b* deletion reduced tumour incidence, but were not able to demonstrate conclusively that it binds 9-HODE or 13-HODE.

In an effort to identify downstream mediators of HODEs and polyunsaturated fatty acids, we treated S2 cells with 9- and 13-HODE, as well as omega-3 and -6 fatty acids, and showed upregulation of several haemocyte differentiation markers, suggesting that HODEs play a role in activating and differentiating cells. Future RNA sequencing of 9- and 13-HODE treated cells will provide a larger repertoire of genes affected by HODEs.

Over-expression and purification of Pxn, Pxt and Irc protein revealed that, these enzymes demonstrate in vitro cyclooxygenase activity, having the ability to utilise AA to produce the prostaglandin precursor PGH₂. We also showed that these enzymes also possess MPO activity, as they were able to use hydrogen peroxide as a substrate. This is compelling evidence to suggest they are able to produce oxygenated lipid inflammatory mediators. In future work, we will assay whether these enzymes are capable of metabolizing linoleic acid to produce HODEs in vitro.

Finally, we have shown that deletion of the COX/MPO homologues, Pxn, Pxt and Irc has significant effects on the survival of *Drosophila* both in response to sterile and septic wounding. Our data is consistent with Pxn, Pxt and Irc producing lipid mediators like HODEs that are fundamental to immune responses and tissue repair.

Although *e* lacks the canonical mammalian inflammatory components, arachidonic acid and prostaglandins, we believe that *Drosophila* can be used as a model to

elucidate the molecular mechanisms and functions of C18 lipid mediators such as HODEs. *Drosophila* provides an ideal system to investigate function of these families of immune-modulatory lipid mediators as they lack C20 lipid mediators, which could obscure the function of C18 mediators in mammalian systems. The work shown here lays the foundation for these investigations.

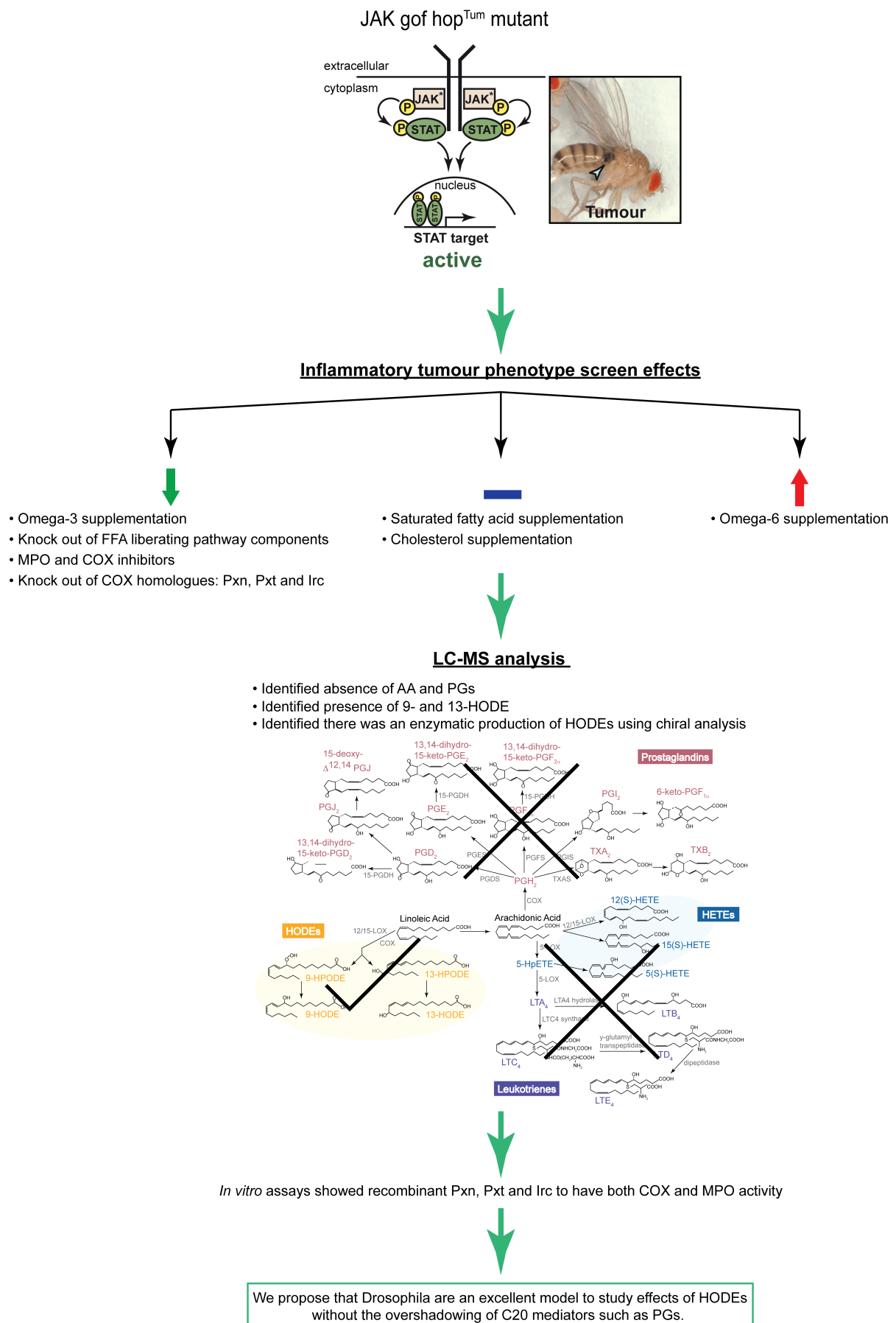


Figure 27. Summary schematic of findings

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